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 NEWS  2           "Ask CAS" for self-help around the clock
 NEWS  3  AUG 09   INSPEC enhanced with 1898-1968 archive
 NEWS  4  AUG 28   ADISCTI Reloaded and Enhanced
 NEWS  5  AUG 30   CA(SM)/CAplus(SM) Austrian patent law changes
 NEWS  6  SEP 11   CA/CAplus enhanced with more pre-1907 records
 NEWS  7  SEP 21   CA/CAplus fields enhanced with simultaneous left and right
                   truncation
 NEWS  8  SEP 25   CA(SM)/CAplus(SM) display of CA Lexicon enhanced
 NEWS  9  SEP 25   CAS REGISTRY(SM) no longer includes Concord 3D coordinates
 NEWS 10  SEP 25   CAS REGISTRY(SM) updated with amino acid codes for pyrrolysine
 NEWS 11  SEP 28   CEABA-VTB classification code fields reloaded with new
                   classification scheme
 NEWS 12  OCT 19   LOGOFF HOLD duration extended to 120 minutes
 NEWS 13  OCT 19   E-mail format enhanced
 NEWS 14  OCT 23   Option to turn off MARPAT highlighting enhancements available
 NEWS 15  OCT 23   CAS Registry Number crossover limit increased to 300,000 in
                   multiple databases
 NEWS 16  OCT 23   The Derwent World Patents Index suite of databases on STN
                   has been enhanced and reloaded
 NEWS 17  OCT 30   CHEMLIST enhanced with new search and display field
 NEWS 18  NOV 03   JAPIO enhanced with IPC 8 features and functionality
 NEWS 19  NOV 10   CA/CAplus F-Term thesaurus enhanced
 NEWS 20  NOV 10   STN Express with Discover! free maintenance release Version
                   8.01c now available

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               MACINTOSH VERSION IS V6.0c(ENG) AND V6.0Jc(JP),
               AND CURRENT DISCOVER FILE IS DATED 25 SEPTEMBER 2006.

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 NEWS X25      X.25 communication option no longer available

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FILE 'HOME' ENTERED AT 00:18:42 ON 13 NOV 2006

=> file uspatful
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                       ENTRY    SESSION
FULL ESTIMATED COST                                     0.21       0.21

FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006
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FILE COVERS 1971 TO PATENT PUBLICATION DATE: 9 Nov 2006 (20061109/PD)
FILE LAST UPDATED: 9 Nov 2006 (20061109/ED)
HIGHEST GRANTED PATENT NUMBER: US7134145
HIGHEST APPLICATION PUBLICATION NUMBER: US2006253949
CA INDEXING IS CURRENT THROUGH 7 Nov 2006 (20061107/UPCA)
ISSUE CLASS FIELDS (/INCL) CURRENT THROUGH: 9 Nov 2006 (20061109/PD)
REVISED CLASS FIELDS (/NCL) LAST RELOADED: Jun 2006
USPTO MANUAL OF CLASSIFICATIONS THESAURUS ISSUE DATE: Jun 2006


=> e garcon natalie/in
E1            1     GARCON JOHNSON NATHALIE MARIE JOSEPH CLAUDE/IN
E2            4     GARCON JOHNSON NATHALIE MARIE JOSEPHE CLAUDE/IN
E3            0 --> GARCON NATALIE/IN
E4           16     GARCON NATHALIE/IN
E5            1     GARCON NATHALIE B/IN
E6            5     GARCON NATHALIE MARIE JOSEPHE/IN
E7            3     GARCON NATHALIE MARIE JOSEPHE CLAUDE/IN
E8            1     GARCON STEPHANIE/IN
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E9          1     GARCON THIERRY/IN
E10         3     GARCONNAT MICHEL/IN
E11         2     GARCONNET CLAUDE/IN
E12         2     GARCONNET DOMINIQUE/IN

=> s e4-e7
            16 "GARCON NATHALIE"/IN
             1 "GARCON NATHALIE B"/IN
             5 "GARCON NATHALIE MARIE JOSEPHE"/IN
             3 "GARCON NATHALIE MARIE JOSEPHE CLAUDE"/IN
L1          25 ("GARCON NATHALIE"/IN OR "GARCON NATHALIE B"/IN OR "GARCON NATHA
               LIE MARIE JOSEPHE"/IN OR "GARCON NATHALIE MARIE JOSEPHE CLAUDE"/
              IN)

=> s l1 and (CpG)
          9147 CPG
L2           8 L1 AND (CPG)

=> d l2,cbib,clm,1-8

L2   ANSWER 1 OF 8  USPATFULL on STN
2005:227456 Vaccines.
    Dalton, Colin Cave, Rixensart, BELGIUM
    Easeman, Richard Lewis, Brentford, UNITED KINGDOM
    Garcon, Nathalie, Rixensart, BELGIUM
    SmithKline Beecham Biologicals s.a. (non-U.S. corporation)SmithKline
    Beecham p.l.c. (non-U.S. corporation)
    US 2005197308 A1 20050908
    APPLICATION: US 2004-17103  A1  20041220 (11)
    PRIORITY: GB 2000-17999  20000721
    GB 2001-21171  20010831
    DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM    What is claimed is:
       1. A pharmaceutical agent delivery device having at least one
       skin-piercing member comprising a solid biodegradable reservoir medium
       containing the pharmaceutical agent.

       2. A pharmaceutical agent delivery device as claimed in claim 1, wherein
       the solid biodegradable reservoir medium containing the pharmaceutical
       agent is coated externally onto at least one skin-piercing member.

       3. A pharmaceutical agent delivery device as claimed in claim 1 wherein
       the solid biodegradable reservoir medium is a polyol.

       4. A pharmaceutical agent delivery device as claimed in claim 3, wherein
       the polyol is a stabilizing polyol.

       5. A pharmaceutical agent delivery device as claimed in claim 1 wherein
       the solid biodegradable reservoir medium is a sugar.

       6. A pharmaceutical agent delivery device as claimed in claim 5 wherein
       the sugar is selected from lactose, sucrose, raffinose or trehalose.

       7. A pharmaceutical agent delivery device as claimed in claim 1 wherein
       the solid biodegradable reservoir medium forms a glass.

       8. A pharmaceutical agent delivery device as claimed in claim 1 wherein
       the solid biodegradable reservoir medium releases the pharmaceutical
       agent within 5 minutes after insertion of the skin-piercing member and
       solid biodegradable reservoir medium into the skin.

       9. A pharmaceutical agent delivery device as claimed in claim 1 wherein
       the skin piercing members are dimensioned to deliver the agent into the
       dermis.

       10. A pharmaceutical agent delivery device as claimed in claim 1 wherein
       the skin piercing members are dimensioned to deliver the agent into the
       epidermis.

       11. A pharmaceutical agent delivery device as claimed in claim 1 wherein
       the skin piercing members microneedles or microblades.

       12. A pharmaceutical agent delivery device as claimed in claim 1 wherein
       the pharmaceutical agent is a vaccine.

       13. A pharmaceutical agent delivery device as claimed in claim 12
       wherein the vaccine comprises an antigen.
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14. A pharmaceutical agent delivery device as claimed in claim 12 wherein the vaccine comprises nucleic acid encoding an antigen.

15. A process for the preparation of a pharmaceutical delivery device comprising making a solution of pharmaceutical agent and reservoir medium, followed by dipping at least one skin-piercing member into said solution, and allowing the solution to dry onto the skin-piercing member to form a solid biodegradable reservoir medium containing the pharmaceutical agent.

16. A skin patch for delivery of vaccines comprising an array of microblades or microneedles coated with a glassy sugar reservoir medium containing the vaccine.

L2 ANSWER 2 OF 8 USPATFULL on STN
2005:22798 Vaccines.
Garcon, Nathalie, Rixensart, BELGIUM
Gerard, Catherine Marie Ghislaine, Rixensart, BELGIUM
Stephenne, Jean, Rixensart, BELGIUM
US 2005019340 A1 20050127
APPLICATION: US 2004-478188 A1 20040805 (10)
WO 2001-EP11984 20011016
PRIORITY: GB 2000-25573 20001018
GB 2000-25574 20001018
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. An immunogenic composition comprising a cancer antigen selected from the group: i) an antigen from the MAGE protein family linked to a heterologous fusion partner; ii) prostase antigen linked to a heterologous fusion partner; iii) prostase fragments optionally linked to a heterologous fusion partner; iv) P501S; v) Cripto; vi) Her-2/neu antigen derivative devoid of a substantial portion of the Her-2/neu transmembrane domain, and an adjuvant composition comprising a saponin, together with an immunostimulatory oligonucleotide.

2. A composition as claimed in claim 1 further comprising a lipopolysaccharide.

3. A composition as claimed in claim 1 wherein the saponin is QS21.

4. A composition as claimed in any of claim 2 or 3 wherein the lipopolysaccharide is selected from the group of i Monophosphoryl Lipid A ii 3-0-Deacylated Monophosphoryl Lipid A iii Disphosphoryl Lipid A

5. An immunogenic composition as claimed in any of claims 1 to 4 wherein the immunostimulatory oligonucleotide contains at least two **CpG** motifs.

6. An immunogenic composition as claimed in any of claims 1 to 5 wherein the immunostimulatory oligonucleotide is selected from the group:

SEQ ID No 1
TCC ATG ACG TTC CTG ACG TT (**CpG** 1826)-

SEQ ID No 2
TCT CCC AGC GTG CGC CAT (**CpG** 1758)-

SEQ ID No 3
ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG-

SEQ ID No 4
TCG TCG TTT TGT CGT TTT GTC GTT (**CpG** 2006)- SEQ ID No 5--TCC ATG ACG TTC CTG ATG CT (**CpG** 1668)

7. A composition as claimed in any of claims 1 to 6 wherein the saponin is formulated to form ISCOMS or liposomes.

8. A composition as claimed in any of claims 1 to 6 wherein the saponin is present in an oil in water emulsion.

9. A composition as claimed in any of claims 1 to 8 comprises substantially all of the extracellular domain of Her 2 neu.

10. A composition as claimed in claim 8 wherein the Her 2 neu molecule is devoid of a functional transmembrane domain.

11. A composition as claimed in claim 1 to 10 which additional comprises the phosphorylation domain of Her 2 neu.

12. A method of treating a patient suffering from or susceptible to, a cancer expressing a Her 2 neu or prostate specific/tumour antigen comprising administering a safe and effective amount of a composition according to any of claims 1 to 11.

13. A method of treating a patient suffering from or susceptible to a cancer expressing any of MAGE, prostase, P501S or Cripto comprising administering a safe and effective amount of a composition according to any of claims 1 to 11.

14. Use of a combination of a saponin an immunostimulatory oligonucleotide and a cancer antigen selected from the group: i) an antigen from the MAGE protein family linked to a heterologous fusion partner; ii) prostase antigen linked to a heterologous fusion partner; iii) prostase fragments optionally linked to a heterologous fusion partner; iv) P501S; v) Cripto; vi) Her-2/neu antigen derivative devoid of a substantial portion of the Her-2/neu transmembrane domain, in the manufacture of a medicament for the treatment or prophylaxis of tumours.

15. A method of manufacture of a composition as claimed in any of claims 1 to 11, comprising admixing a cancer antigen selected from the group: i) an antigen from the MAGE protein family linked to a heterologous fusion partner; ii) prostase antigen linked to a heterologous fusion partner; iii) prostase fragments optionally linked to a heterologous fusion partner; iv) P501S; v) Cripto; vi) Her-2/neu antigen derivative devoid of a substantial portion of the Her-2/neu transmembrane domain, with a saponin and **CpG** molecule.

L2 ANSWER 3 OF 8 USPATFULL on STN
2005:3856 Vaccines.
Cohen, Joseph, Rixensart, BELGIUM
Garcon, Nathalie, Rixensart, BELGIUM
Voss, Gerald, Rixensart, BELGIUM
SmithKline Beecham Biologicals SA (non-U.S. corporation)
US 2005002958 A1 20050106
APPLICATION: US 2004-789758 A1 20040227 (10)
PRIORITY: GB 1999-15204 19990629
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1-12. Cancelled

13. A composition for raising an immune response comprising a malaria antigen and an immunostimulatory **CpG** oligonucleotide.

14. A composition as claimed in claim 13 wherein the antigen is selected from the group of malaria antigens consisting of RTS, RTS*, TRAP and immunologically equivalent derivatives thereof.

15. A composition as claimed in claim 13 wherein the vaccine comprises TRAP or immunologically equivalent derivative and one of RTS or RTS*.

16. A composition as claimed in claim 13 further comprising an aluminum salt, 3 de-O-acylated monophosphoryl lipid A or a saponin adjuvant.

17. A composition as claimed in claim 13 wherein the oligonucleotide comprises two **CpG** dinucleotides.

18. A composition as claimed in claim 13 wherein the **CpG** oligonucleotide is between 15-45 nucleotides in length.

19. A composition as claimed in claim 13 wherein the **CpG** oligonucleotide comprises at least one phosphorothioate internucleotide bond.

20. A composition as claimed in claim 13 wherein the oligonucleotide is selected from the group consisting of oligonucleotides designated as WD1001, WD1002, WD1003, WD1004, WD1005, WD1006, and WD1007.

21. A method for the prevention or amelioration of plasmodium infection in a patient, comprising administering an effective amount of a composition of claim 13 to a patient.

22. A method for the prevention or amelioration of plasmodium infection in a patient, comprising administering an effective amount of a composition of claim 16 to a patient.

23. A method of producing a composition as claimed in claim 13 comprising admixing a malarial antigen and a **CpG** immunostimulatory oligonucleotide.

24. A method for the prevention or amelioration of plasmodium infection in a patient, comprising administering an effective amount of a **CpG** oligonucleotide followed after a suitable time by an effective amount of a malaria antigen.

L2 ANSWER 4 OF 8 USPATFULL on STN
2004:164903 Vaccine composition.
Berthet, Francois-Xavier Jacques, Rixensart, BELGIUM
Dalemans, Wilfried L J, Rixensart, BELGIUM
Denoel, Philippe, Rixensart, BELGIUM
Dequesne, Guy, Rixensart, BELGIUM
Feron, Chriatiane, Rixensart, BELGIUM
Garcon, Nathalie, Rixensart, BELGIUM
Lobet, Yves, Rixensart, BELGIUM
Poolman, Jan, Rixensart, BELGIUM
Thiry, Georges, Rixensart, BELGIUM
Thonnard, Joelle, Rixensart, BELGIUM
Voet, Pierre, Rixensart, BELGIUM
US 2004126389 A1 20040701
APPLICATION: US 2003-343561 A1 20030915 (10)
WO 2001-EP8857 20010731
PRIORITY: GB 2001-3170 20010208
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. An immunogenic composition comprising an antigen derived from a pathogen which is capable of protecting a host against said pathogen, mixed with an adjuvant comprising a bleb preparation derived from a Gram-negative bacterial strain, with the proviso that an immunogenic composition consisting of N. meningitidis B blebs and N. meningitidis C polysaccharide antigen is not claimed.

2. The immunogenic composition comprising an antigen comprising 1 or more conjugated meningococcal capsular polysaccharides selected from a group comprising: A, Y or W, mixed with an adjuvant comprising a bleb preparation from meningoccocus B.

3. The immunogenic composition of claim 1, wherein the antigen and the Gram-negative bacterial bleb preparation are from different pathogens.

4. The immunogenic composition of claim 3, wherein the antigen is a conjugated capsular polysaccharide from H. influenzae b, and the bleb preparation is from meningoccocus B.

5. The immunogenic composition of claim 3, wherein the antigen is one or more conjugated capsular polysaccharide(s) from Streptococcus pneumoniae selected from the group consisting of: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F, and the bleb preparation is from meningoccocus B.

6. The immunogenic composition of claim 2, 4 or 5, wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: htrB, msbB and lpxK.

7. The immunogenic composition of claim 2, 4, 5 or 6, wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level one or more genes selected from the group consisting of: pmrA, pmrB, pmrE and pmrF.

8. The immunogenic composition of claim 2, 4, 5, 6 or 7, wherein the bleb preparation is derived from a strain which does not produce B capsular polysaccharide, due to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: galE, siaA, siaB, siaC, siaD, ctrA, ctrB, ctrC and ctrD.

9. The immunogenic composition of claim 3, wherein the antigen is from H. influenzae, and the bleb preparation is from Moraxella catarrhalis.

10. The immunogenic composition of claim 9, wherein the antigen is a

conjugated capsular polysaccharide from H. influenzae b.

11. The immunogenic composition of claim 3, wherein the antigen is from Streptococcus pneumoniae, and the bleb preparation is from Moraxella catarrhalis.

12. The immunogenic composition of claim 11, wherein the antigen is one or more conjugated capsular polysaccharide(s) from Streptococcus pneumoniae selected from the group consisting of: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.

13. The immunogenic composition of claim 11, wherein the antigen is one or more proteins from Streptococcus pneumoniae capable of protecting a host against pneumococcal disease.

14. The immunogenic composition of claims 9-13, wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: htrB, msbB and lpxK.

15. The immunogenic composition of claims 9-14, wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level one or more genes selected from the group consisting of: pmrA, pmrB, pmrE and pmrF.

16. The immunogenic composition of claim 3, wherein the antigen is a conjugated capsular polysaccharide from H. influenzae b, and the bleb preparation is from non-typeable H. influenzae.

17. The immunogenic composition of claim 3, wherein the antigen is from Streptococcus pneumoniae, and the bleb preparation is from non-typeable H. influenzae.

18. The immunogenic composition of claim 17, wherein the antigen is one or more conjugated capsular polysaccharide(s) from Streptococcus pneumoniae selected from the group consisting of: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.

19. The immunogenic composition of claim 17, wherein the antigen is one or more proteins from Streptococcus pneumoniae capable of protecting a host against pneumococcal disease.

20. The immunogenic composition of claim 3, wherein the antigen is from Moraxella catarrhalis, and the bleb preparation is from non-typeable H. influenzae.

21. The immunogenic composition of claim 20, wherein the antigen is one or more proteins from Moraxella catarrhalis capable of protecting a host against disease caused by Moraxella catarrhalis.

22. The immunogenic composition of claims 13-21, wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: htrB, msbB and lpxK.

23. The immunogenic composition of claims 13-22, wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level one or more genes selected from the group consisting of: pmrA, pmrB, pmrE and pmrF.

24. A vaccine comprising the immunogenic composition of claims 1-23, and a pharmaceutically acceptable excipient or carrier.

25. A method of inducing a faster protective immune response against the antigen contained in the immunogenic composition of claims 1-23, comprising the step of administering to a host an effective amount of the immunogenic composition of claims 1-23.

26. A method of inducing an enhanced immune response against the antigen contained in the immunogenic composition of claims 1-23, comprising the step of administering to a host an effective amount of the immunogenic composition of claims 1-23.

27. A method of protecting an elderly patient against a pathogen by administering to said patient an effective amount of the immunogenic composition of claims 1-23 in which the antigen is derived from said pathogen.

28. Use of the immunogenic preparation of claims 1-23 in the manufacture of a medicament for the treatment of a disease caused by the pathogen from which the antigen is derived.

29. Use of bleb derived from Moraxella catarrhalis as an adjuvant in an immunogenic composition comprising one or more pneumococcal capsular polysaccharides.

30. Use of bleb derived from Moraxella catarrhalis as an adjuvant in an immunogenic composition comprising one or more pneumococcal protein antigens.

31. Use of bleb derived from non-typeable H. influenzae as an adjuvant in an immunogenic composition comprising one or more pneumococcal capsular polysaccharides.

32. Use of bleb derived from non-typeable H. influenzae as an adjuvant in an immunogenic composition comprising one or more pneumococcal protein antigens.

33. A process for making an immunogenic composition comprising the step of mixing an antigen derived from a pathogen which is capable of protecting a host against said pathogen, with an adjuvant comprising a bleb preparation derived from a Gram-negative bacterial strain.

L2 ANSWER 5 OF 8 USPATFULL on STN
2004:64631 Vaccines.
Dalton, Colin Cave, Rixensart, BELGIUM
Easeman, Richard Lewis, Brentford, UNITED KINGDOM
Garcon, Nathalie, Rixensart, BELGIUM
US 2004049150 A1 20040311
APPLICATION: US 2003-333448 A1 20030812 (10)
WO 2001-EP8339 20010718
PRIORITY: GB 2000-17999 20000721
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. A pharmaceutical agent delivery device having at least one skin-piercing member comprising a solid biodegradable reservoir medium containing the pharmaceutical agent.

2. A pharmaceutical agent delivery device as claimed in claim 1, wherein the solid biodegradable reservoir medium containing the pharmaceutical agent is coated externally onto the at least one skin-piercing member.

3. A pharmaceutical agent delivery device as claimed in claims 1 or 2 wherein the solid biodegradable reservoir medium is a polyol.

4. A pharmaceutical agent delivery device as claimed in claim 3, wherein the polyol is a stabilising polyol.

5. A pharmaceutical agent delivery device as claimed in any one of claims 1 to 4 wherein the solid biodegradable reservoir medium is a sugar.

6. A pharmaceutical agent delivery device as claimed in claim 5 wherein the sugar is selected from lactose, sucrose, raffinose or trehalose.

7. A pharmaceutical agent delivery device as claimed in any one of claims 1 to 5 wherein the solid biodegradable reservoir medium forms a glass.

8. A pharmaceutical agent delivery device as claimed in any on of claims 1 to 7 wherein the solid biodegradable reservoir medium releases the pharmaceutical agent within 5 minutes after insertion of the skin-piercing member and solid biodegradable reservoir medium into the skin.

9. A pharmaceutical agent delivery device as claimed in any one of claims 1 to 8 wherein the skin piercing members are dimensioned to deliver the agent into the dermis.

10. A pharmaceutical agent delivery device as claimed in any one of

claims 1 to 8 wherein the skin piercing members are dimensioned to deliver the agent into the epidermis.

11. A pharmaceutical agent delivery device as claimed in any one of claims 1 to 10 wherein the skin piercing members microneedles or microblades.

12. A pharmaceutical agent delivery device as claimed in any one of claims 1 to 11, wherein the pharmaceutical agent is a vaccine.

13. A pharmaceutical agent delivery device as claimed in claim 12 wherein the vaccine comprises an antigen.

14. A pharmaceutical agent delivery device as claimed in claim 12 wherein the vaccine comprises nucleic acid encoding an antigen.

15. A process for the preparation of a pharmaceutical delivery device comprising making a solution of pharmaceutical agent and reservoir medium, followed by dipping at least one skin-piercing member into said solution, and allowing the solution to dry onto the skin-piercing member to form a solid biodegradable reservoir medium containing a the pharmaceutical agent.

16. A skin patch for delivery of vaccines comprising an array of microblades or microneedles coated with a glassy sugar reservoir medium containing the vaccine

L2 ANSWER 6 OF 8 USPATFULL on STN
2004:63351 Adjuvant composition comprising an immunostimulatory oligonucleotide and a tocol.
Garcon, Nathalie, Rixensart, BELGIUM
Gerard, Catherine Marie Ghislaine, Rixensart, BELGIUM
Stephenne, Jean, Rixensart, BELGIUM
US 2004047869 A1 20040311
APPLICATION: US 2003-399356 A1 20030930 (10)
WO 2001-EP11985 20011016
PRIORITY: GB 2000-255778 20001018
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. An adjuvant composition comprising a combination of an immunostimulatory oligonucleotide and a tocol.

2. An adjuvant composition as claimed in claim 1 wherein the tocol is in the form of an oil in water emulsion.

3. An adjuvant composition as claimed in claim 2 wherein the oil in water emulsion further comprises squalene.

4. An adjuvant composition as claimed in any one of claims 1 to 3, wherein said immunostimulatory oligonucleotide comprises a Purine, Purine, C, G, pyrimidine, pyrimidine sequence, wherein the C and G are unmethylated.

5. An adjuvant composition as claimed in claims 1 to 3, wherein said immunostimulatory oligonucleotide is selected from the group comprising: TCC ATG ACG TTC CTG ACG TT (SEQ ID NO:1); TCT CCC AGC GTG CGC CAT (SEQ ID NO:2); ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG (SEQ ID NO:3); TCG TCG TTT TGT CGT TTT GTC GTT (SEQ ID NO:4); TCC ATG ACG TTC CTG ATG CT (SEQ ID NO:5).

6. An adjuvant composition according to claim 1 to 3, wherein the immunostimulatory oligonucleotide contains at least two unmethylated CG repeats being separated at least by 3 nucleotides.

7. An adjuvant composition according to claim 6, wherein the immunostimulatory oligonucleotide contains at least two unmethylated CG repeats being separated by 6 nucleotides.

8. An adjuvant composition as claimed in claim 1 wherein the tocol is described by the general formula: ##STR5## wherein R may be H or one or more identical or different substituents chosen from the group comprising alkyl, alkoxy, acyloxy, hydroxy, a sulphate and a phosphate group; R1 and R3 independently of one another are H or alkyl; R2 is H or alkyl and may be different in each unit; the broken line indicates the presence or absence of an additional carbon-carbon bond in a unit; and n=the value 1 to 10. The alkyl group in R, R1, R2 and R3 may be chosen in particular from a linear or branched carbon chain having 1-4

carbon atoms, such as methyl, ethyl, butyl or isobutyl.

9. An adjuvant composition as claimed in claim 8, wherein the tocol is D, L, α-tocopherol.

10. An adjuvant composition as claimed in any one of claims 1 to 9, wherein said adjuvant further comprises an additional immunostimulant.

11. An adjuvant composition as claimed in claim 10 wherein the additional immunostimulant is selected from LPS or a derivative thereof, 3D-MPL, a saponin, or QS21.

12. A vaccine composition comprising an adjuvant composition as claimed in any one of claims 1 to 11, and an antigen or antigenic composition.

13. A vaccine as claimed in claim 12, wherein the antigen is ECD-PD.

14. A method of shifting the quality of an immune response against an antigen, generated by a vaccine comprising an immunostimulatory oligonucleotide, towards a Th1-type immune response, the method comprising formulating the vaccine with an immunostimulatory oligonucleotide and a tocol containing oil in water emulsion.

15. A method of shifting the quality of an immune response as claimed in claim 14, wherein the combination of the immunostimulatory oligonucleotide with a tocol containing oil in water emulsion generates a Th1-type immune response such that when antigen specific IgG isotypes induced by the vaccine after vaccination of a mouse are measured, IgG1 constitutes less than 50% of the total antigen specific IgG as determined by mid point titres measured by isotype specific ELISA.

16. A method of manufacturing a vaccine composition comprising formulating an oil in water emulsion comprising a tocol, admixing said tocol emulsion with an immunostimulatory oligonucleotide to form an adjuvant, and formulating said adjuvant with an antigen or antigenic composition.

17. A method of treating an individual susceptible to or suffering from a disease comprising the administration to said individual of a vaccine composition comprising a combination of an immunostimulatory oligonucleotide and a tocol.

18. An vaccine as claimed in claim 12 for use in medicine.

L2 ANSWER 7 OF 8 USPATFULL on STN
2003:231636 Vaccines.
Friede, Martin, Farnham, UNITED KINGDOM
Garcon, Nathalie, Wavre, BELGIUM
Gerard, Catherine Marie Ghislaine, Rhode Saint Genese, BELGIUM
Hermand, Philippe, Court-Saint-Etienne, BELGIUM
SmithKline Beecham Biologicals s.a. (non-U.S. corporation)
US 2003161834 A1 20030828
APPLICATION: US 2003-379164 A1 20030303 (10)
PRIORITY: GB 1999-8885 19990419
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. An adjuvant composition comprising a saponin and an immunostimulatory oligonucleotide.

2. An adjuvant composition according to claim 1 further comprising a carrier.

3. An adjuvant composition as claimed in claim 1 or 2, wherein said saponin is selected from the group comprising Quil A, or purified saponins such as QS21, QS7, QS17; -escin, or digitonin.

4. An adjuvant composition as claimed in any one of claims 1 to 3, wherein said immunostimulatory oligonucleotide comprises a Purine, Purine, C, G, pyrimidine, pyrimidine sequence.

5. An adjuvant composition as claimed in claims 1 to 4, wherein said immunostimulatory oligonucleotide is selected from the group comprising: TCC ATG ACG TTC CTG ACG TT (SEQ ID NO: 1); TCT CCC AGC GTG CGC CAT (SEQ ID NO: 2); ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG (SEQ ID NO: 3); TCG TCG TTT TGT CGT TTT GTC GTT (SEQ ID NO: 4); TCC ATG ACG TTC CTG ATG CT (SEQ ID NO: 5).

6. An adjuvant composition according to claim 1 to 4, wherein the immunostimulatory oligonucleotide contains at least two unmethylated CG repeats being separated at least by 3 nucleotides.

7. An adjuvant composition according to claim 6, wherein the immunostimulatory oligonucleotide contains at least two unmethylated CG repeats being separated by 6 nucleotides.

8. An adjuvant composition as claimed in any one of claims 2 to 7, wherein said carrier is a particulate carrier selected from the group comprising mineral salts, emulsions, polymers, liposomes, ISCOMs.

9. A vaccine composition comprising an adjuvant composition as claimed in any one of claims 1 to 8, further comprising an antigen.

10. A vaccine composition as claimed in claim 9, wherein said antigen is derived from an organism selected from the group comprising: Human Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Dengue virus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia. Bordetella, Streptococcus, Mycoplasma, Mycobacteria, Haemophilus, Plasmodium or Toxoplasma, stanworth decapeptide; or Tumour associated antigens (TAA), MAGE, BAGE, GAGE, MUC-1, Her-2 neu, CEA, PSA, KSA, or PRAME; or a self peptide hormone, GnRH.

11. A vaccine composition as claimed in claim 9, wherein said antigen is derived from the group comprising (a) tumour associated antigens PSMA, PSCA, tyrosinase, survivin, NY-ESO1, prostase, PS108, RAGE, LAGE, IIAGE; (b) or the N terminal 39-43 amino acid fragment (A f the amyloid precursor protein; (c) or antigens associated to atherosclerosis.

12. A vaccine composition as claimed in claims 9 to 11 wherein the vaccine is administered systemically.

13. A vaccine composition as claimed in claims 9 to 11 wherein the vaccine is administered mucosally.

14. A vaccine composition as claimed in claim 13 wherein the saponin of the adjuvant composition is haemolytic.

15. A delivery device pre-filled with the vaccine of claims 9 to 11, said device being designed to administer the vaccine systemically.

16. A method of inducing an immune response in an individual, comprising the systemic administration of a safe and effective amount of the vaccine composition as claimed in claims 9 to 11.

17. A method of treatment of an individual susceptible to or suffering from a disease by the administration to an individual of an effective amount of the vaccine as claimed in any one of claims 9 to 14.

18. A method of treatment as claimed in claim 17, wherein the administration of the vaccine is through a systemic route.

19. A method of treatment of an individual suffering from a disease selected from the group comprising prostate, breast, colorectal, lung, pancreatic, renal, ovarian or melanoma cancers; noncancer chronic disorders, allergy, Alzheimer, atherosclerosis, comprising the administration of a vaccine as claimed in any one of claims 9 to 11.

20. A method for preventing an individual suffering from contracting a disease selected from the group comprising prostate, breast, colorectal, lung, pancreatic, renal, ovarian or melanoma caners; non-cancer chronic disorders, allergy, Alzheimer, atherosclerosis, comprising the administration of a vaccine as claimed in any one of claims 9 to 11.

21. A method of treatment as claimed in claims 19 and 20, wherein the vaccine is administered via a systemic route.

22. A vaccine as claimed in claim 9 or 11 for use as a medicament.

23. Use of a combination of a saponin and a CpG molecule in the manufacture of a vaccine for the prophylaxis and the treatment of viral, bacterial, parasitic infections, allergy, cancer or other chronic disorders.

24. Use of combination of a saponin, an inmmunostimulatory

oligonucleotide and a carrier in the manufacture of a vaccine for the prophylaxis and the treatment of viral, bacterial, parasitic infections, allergy, cancer or other chronic disorders.

25. A method of inducing a systemic antigen specific immune response in a mammal, comprising administering to a mucosal surface of said mammal a composition comprising an antigen and a haemolytic saponin and a **CpG** molecule.

26. Method of making an adjuvant composition comprising admixing a saponin with an immunostimulatory oligonucleotide.

27. Method of making an adjuvant composition comprising admixing a saponin, an immunostimulatory oligonucleotide, and a carrier.

28. Method of making a vaccine comprising admixing the following (a) a saponin, (b) an immnunostimulatory oligonucleotide, and (c) an antigen.

29. Method of making a vaccine comprising admixing the following (a) a saponin, (b) an immunostimulatory oligonucleotide, (c) a carrier and (d) an antigen.

L2 ANSWER 8 OF 8 USPATFULL on STN
2003:95812 Vaccines.
Friede, Martin, Farnham, UNITED KINGDOM
Garcon, Nathalie, Wavre, BELGIUM
Gerard, Catherine Marie Ghislaine, Rhode Saint Genese, BELGIUM
Hermand, Philippe, Court-Saint-Etienne, BELGIUM
SmithKline Beecham Biologicals s.a., Rixensart, BELGIUM (non-U.S. corporation)
US 6544518 B1 20030408
APPLICATION: US 2000-690921 20001018 (9)
PRIORITY: GB 1999-8885 19990419
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. An adjuvant composition comprising a QS21 and an immunostimulatory oligonucleotide containing an unmethylated CG dinucleotide.

2. An adjuvant composition according to claim 1 further comprising a carrier.

3. An adjuvant composition as claimed in claim 1, wherein said immunostimulatory oligonucleotide comprises a Purine, Purine, C, G, pyrimidine, pyrimidine sequence.

4. An adjuvant composition as claimed in claim 1, wherein said immunostimulatory oligonucleotide is selected from the group comprising: TCC ATG ACG TTC CTG ACG TT (SEQ ID NO:1); TCT CCC AGC GTG CGC CAT (SEQ ID NO:2); ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG (SEQ ID NO:3); TCG TCG TTT TGT CGT TTT GTC GTT (SEQ ID NO:4); TCC ATG ACG TTC CTG ATG CT (SEQ ID NO:5).

5. An adjuvant composition as claimed in claim 1, wherein the immunostimulatory oligonucleotide contains at least two unmethylated CG repeats being separated at least by 3 nucleotides.

6. An adjuvant composition according to claim 5, wherein the immunostimulatory oligonucleotide contains at least two unmethylated CG repeats being separated by 6 nucleotides.

7. An adjuvant composition as claimed in claim 2, wherein said carrier is a particulate carrier selected from the group comprising metallic salt particles, emulsions, polymers, liposomes, ISCOMs.

8. An immunogenic composition comprising an adjuvant composition as claimed in claims 1 or 2, further comprising an antigen.

9. An immunogenic composition as claimed in claim 8, wherein said antigen is derived from an organism selected from the group comprising: Human Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Dengue virus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Streptococcus, Mycoplasma, Mycobacteria, Haemophilus, Plasmodium or Toxoplasma, stanworth decapeptide; or the N terminal 39-43 amino acid fragment (Abeta) of the amyloid precursor protein or antigens associated with atheroschlerosis.

10. An immunogenic composition as claimed in claim 8 wherein the vaccine is administered systemically.

11. An immunogenic composition as claimed in claim 8 wherein the vaccine is administered mucosally.

12. A delivery device pre-filled with the immunogenic composition of claim 8, said device being designed to administer the immunogenic composition systemically.

13. An adjuvant composition according to claim 1 or 2, wherein QS21 is in the form of a liposome.

14. An adjuvant composition according to claim 1 or 2, wherein QS21 is in the form of an oil in water emulsion.

15. An adjuvant composition as claimed in claim 7, wherein the metallic salt particle is aluminium hydroxide or aluminium phosphate.

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=> d his

     (FILE 'HOME' ENTERED AT 00:18:42 ON 13 NOV 2006)

     FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006
               E GARCON NATALIE/IN
L1            25 S E4-E7
L2             8 S L1 AND (CPG)

=> s l1 not l2
L3            17 L1 NOT L2

=> d l3,ti,1-17

L3   ANSWER 1 OF 17  USPATFULL on STN
TI     Vaccines

L3   ANSWER 2 OF 17  USPATFULL on STN
TI     Vaccine composition

L3   ANSWER 3 OF 17  USPATFULL on STN
TI     Novel vaccine

L3   ANSWER 4 OF 17  USPATFULL on STN
TI     Vaccines containing a saponin and a sterol

L3   ANSWER 5 OF 17  USPATFULL on STN
TI     Novel vaccine

L3   ANSWER 6 OF 17  USPATFULL on STN
TI     Novel vaccine

L3   ANSWER 7 OF 17  USPATFULL on STN
TI     Vaccines

L3   ANSWER 8 OF 17  USPATFULL on STN
TI     Vaccines

L3   ANSWER 9 OF 17  USPATFULL on STN
TI     Oil in water emulsions containing saponins

L3   ANSWER 10 OF 17  USPATFULL on STN
TI     Vaccine composition

L3   ANSWER 11 OF 17  USPATFULL on STN
TI     Vaccine comprising an iscom consisting of sterol and saponin which is
       free of additional detergent

L3   ANSWER 12 OF 17  USPATFULL on STN
TI     Hepatitis B vaccine

L3   ANSWER 13 OF 17  USPATFULL on STN
TI     Vaccines

L3   ANSWER 14 OF 17  USPATFULL on STN
TI     Vaccines
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L3 ANSWER 15 OF 17 USPATFULL on STN
TI Vaccines

L3 ANSWER 16 OF 17 USPATFULL on STN
TI Hepatitis B vaccine

L3 ANSWER 17 OF 17 USPATFULL on STN
TI Liposomes that provide thymic dependent help to weak vaccine antigens

=> d l3,cbib,clm,1-17

L3 ANSWER 1 OF 17 USPATFULL on STN
2006:214600 Vaccines.
Momin, Patricia Marie, Brussels, BELGIUM
Garcon, Nathalie Marie-Josephe, Wavre, BELGIUM
SmithKline Beecham Biologicals, S.A. (non-U.S. corporation)
US 2006182753 A1 20060817
APPLICATION: US 2005-200601 A1 20050810 (11)
PRIORITY: GB 1993-26253 19931223
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1-27. (canceled)

28. A composition comprising an antigen and/or an antigenic composition, 3D-MPL, and an adjuvant in the form of an oil in water emulsion, which adjuvant contains a metabolizable oil, alpha tocopherol, and polyoxyethylene sorbitan monoleate.

29. The composition of claim 28 wherein the antigen and/or antigenic composition is derived from the group consisting of Herpes Simplex Virus type 1, Herpes Simplex Virus type 2, Human cytomegalovirus, Hepatitis A, B, C or E, respiratory Syncytial Virus, Human Papilloma Virus, Influenza Virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium and Toxoplasma, Feline Immunodeficiency Virus, and Human Immunodeficiency Virus.

30. The composition of claim 28 wherein the metabolizable oil is squalene.

31. The composition of claim 30 wherein the ratio of squalene:alpha tocopherol is equal to or less than 1.

32. The composition of claim 28 which further comprises QS21.

33. The composition of claim 28 wherein the oil in water emulsion comprises from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% polyoxyethylene sorbitan monooleate.

34. A method for treating a mammal having or susceptible to a viral, bacterial, or parasitic infection by administering a therapeutically safe and effective amount of an immunogenic composition comprising an antigen and/or antigenic composition, 3D-MPL, and an adjuvant in the form of an oil in water emulsion, which adjuvant contains a metabolizable oil, alpha tocopherol, and polyoxyethylene sorbitan monoleate.

35. An immunogenic composition comprising an antigen and/or an antigenic composition, 3D-MPL, and an adjuvant in the form of an oil in water emulsion, which adjuvant contains a metabolizable oil, alpha tocopherol, and polyoxyethylene sorbitan monoleate.

36. The composition of claim 35 wherein the antigen or antigenic composition is derived from the group consisting of Herpes Simplex Virus type 1, Herpes Simplex Virus type 2, Human cytomegalovirus, Hepatitis A, B, C or E, respiratory Syncytial Virus, Human Papilloma Virus, Influenza Virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium and Toxoplasma, Feline Immunodeficiency Virus, and Human Immunodeficiency Virus.

37. The composition of claim 35 wherein the metabolizable oil is squalene.

38. The composition of claim 37 wherein the ratio of squalene:alpha tocopherol is equal to or less than 1.

39. The composition of claim 35 which further comprises QS21.

40. The composition of claim 35 wherein the oil in water emulsion contains from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% polyoxyethylene sorbitan monooleate.

L3 ANSWER 2 OF 17 USPATFULL on STN
2006:143545 Vaccine composition.
Garcon, Nathalie Marie-Josephe, Rixensart, BELGIUM
Lemoine, Dominique, Rixensart, BELGIUM
Wauters, Florence Emilie Jeanne Francoise, Rixensart, BELGIUM
US 2006121059 A1 20060608
APPLICATION: US 2004-560513 A1 20040614 (10)
WO 2004-EP6426 20040614 20051213 PCT 371 date
PRIORITY: GB 2003-13916 20030616
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. An immunogenic composition comprising a capsular polysaccharide or oligosaccharide of Haemophilus influenzae B (PRP), and a polyanionic polymer.

2. The immunogenic composition of claim 1, wherein PRP is conjugated to a carrier protein which is a source of T-helper cell epitopes.

3. The immunogenic composition of claim 2, wherein the carrier protein is selected from the group consisting of: tetanus toxoid, diphtheria toxoid, CRM197, and protein D.

4. The immunogenic composition of claim 1, the polyanionic polymer having anionic constitutional repeating units.

5. The immunogenic composition of claim 4 4, wherein the polyanionic polymer comprises anionic constitutional repeating units obtained from a group consisting of: acrylic acid, methacrylic acid, maleic acid, fumaric acid, ethylsulphonic acid, vinylsulphuric acid, vinylsulphonic acid, styrenesulphonic acid, vinylphenylsulphuric acid, 2-methacryloyloxyethane sulphonic acid, 3- methacryloyloxy-2-hydroxypropanesulphonic acid, 3-methacryl amido-3-methylbutanoic acid, acrylamidomethylpropanesulfonic acid, vinylphosphoric acid, 4-vinylbenzoic acid, 3-vinyl oxypropane-1-sulphonic acid, N-vinylsuccinimidic acid, and salts of the foregoing.

6. The immunogenic composition of claim 1, wherein the polyanionic polymer is an oligo- or poly-saccharide such as dextran.

7. The immunogenic composition of claim 1 4, wherein the polyanionic polymer is an oligo- or poly-peptide and comprises anionic constitutional repeating units obtained from a group consisting of: L-aspartic acid, D-aspartic acid, L-glutamic acid, D-glutamic acid, and salts of the foregoing.

8. The immunogenic composition of claim 7, wherein the polyanionic polymer is an oligo- or poly-peptide which has a monomer content of no less than 30, 40, 50, 60, 70, 80, 90 or 100% L-aspartic acid and/or L-glutamic acid.

9. The immunogenic composition of claim 7, wherein the oligo- or polypeptide consists of, on average, 5-200 residues.

10. The immunogenic composition of claim 1, wherein the polyanionic polymer is polyanionic heteropolymer.

11. The immunogenic composition of claim 10, wherein the polyanionic heteropolymer consists of two distinct anionic constitutional repeating units.

12. The immunogenic composition of claim 1, wherein the polyanionic polymer is a polyanionic homopolymer.

13. The immunogenic composition of claim 12, wherein the polyanionic polymer is poly-L-glutamic acid (PLG).

14. The immunogenic composition of claim 1, wherein the result of multiplying the concentration of the polyanionic polymer (in μM) by the net negative charge of the polyanionic polymer at pH 7.0 divided by the amount of PRP present in a 0.5 mL dose of the immunogenic composition (in μg) is 300-6000.

15. The immunogenic composition of claim 14, wherein the concentration

of the polyanionic polymer in the composition is 30-2000 in μM.

16. The immunogenic composition of claim 14, wherein the polyanionic polymer has a net negative charge at pH 7.0, on average, of at least 8.

17. The immunogenic composition of claim 16, wherein the polyanionic polymer has at least on average 1 net negative charge at pH 7.0 per 3 monomers.

18. The immunogenic composition of claim 15, wherein the amount of PRP present in a 0.5 mL dose of the immunogenic composition is 1-20 μg.

19. The immunogenic composition of claim 1, wherein the immunogenic composition comprises one or more further antigens.

20. The immunogenic composition of claim 19, wherein the one or more further antigens comprise one or more meningococcal capsular oligosaccharide or polysaccharide-carrier protein conjugates selected from a group consisting of: MenC, MenY, MenA and MenW.

21. The immunogenic composition of claim 19, wherein the one or more further antigens comprise one or more pneumococcal capsular oligosaccharide or polysaccharide-carrier protein conjugates.

22. The immunogenic composition of claim 20, wherein the carrier protein is selected from the group consisting of: tetanus toxoid, diphtheria toxoid, CRM197, and protein D.

23. The immunogenic composition of claim 19, wherein the one or more further antigens comprise tetanus toxoid, diphtheria toxoid, and inactivated whole-cell B. pertussis or one or more acellular B. pertussis antigens.

24. The immunogenic composition of claim 23, wherein the one or more further antigens comprise one or more acellular B. pertussis antigens selected from the group consisting of: pertussis toxiod, FHA, pertactin, agglutinogen 2 and agglutinogen 3.

25. The immunogenic composition of claim 19, wherein the one or more further antigens comprise either or both of Inactivated Polio Vaccine (IPV) and Hepatitis B surface antigen, wherein Hepatitis B surface antigen is preferably adsorbed onto aluminium phosphate.

26. The immunogenic composition of claim 19, which further comprises an adjuvant with a zero point charge greater than 8; wherein the polyanionic polymer prevents flocculation between the adjuvant and PRP and/or reduces the immunological interference that the adjuvant has on PRP.

27. The immunogenic composition of claim 26, wherein the adjuvant is selected from the group consisting of: alum and aluminium hydroxide.

28. The immunogenic composition of claim 26, wherein the adjuvant is present in the immunogenic composition in the amount of 100-1000 μg per 0.5 mL dose.

29. The immunogenic composition of claim 26, wherein at least one of the one or more further antigens is adsorbed onto the adjuvant.

30. The immunogenic composition of claim 29, wherein the presence of the polyanionic polymer does not cause significant desorption of the one or more further antigens adsorbed onto the adjuvant.

31. The immunogenic composition of claim 29, comprising the following antigens adsorbed onto aluminium hydroxide: diphtheria toxoid, tetanus toxoid, pertussis toxoid, FHA and pertactin.

32. The immunogenic composition of claim 31, further comprising unadsorbed IPV and/or Hepatitis B surface antigen adsorbed onto aluminium phosphate.

33. The immunogenic composition of claim 1, which is lyophilised and further comprises a stabilizing excipient selected from the group consisting of: glucose, maltulose, iso-maltulose, lactulose, sucrose, sorbitol, maltose, lactose, iso-maltose, maltitol, lactitol, palatinit, trehalose, raffinose, stachyose, and melezitose.

34. A vaccine comprising the immunogenic composition of claim 19 and a pharmaceutically acceptable excipient.

35. A method of preventing or treating H. influenzae B disease comprising the steps of administering a pharmaceutically effective amount of the vaccine of claim 19 to a patient in need thereof.

36. (canceled)

37. A method to reduce the immunological interference of a Haemophilus influenzae B capsular polysaccharide or oligosaccharide (PRP), preferably conjugated, in a combination vaccine comprising one or more further antigens adsorbed to an adjuvant with a zero point charge greater than 8, wherein such method comprises the steps of: (i) adsorbing the one or more further antigens onto the adjuvant; (ii) adding a polyanionic polymer to said one or more further antigens; and (iii) then adding an immunogenic composition comprising PRP to said one or more further antigens.

38. (canceled)

39. A method to reduce the immunological interference of a Haemophilus influenzae B capsular polysaccharide or oligosaccharide (PRP), preferably conjugated, in a combination vaccine comprising one or more further antigens adsorbed to an adjuvant with a zero point charge greater than 8, wherein such method comprises the steps of: (i) adsorbing the one or more further antigens onto the adjuvant; and (ii) adding an immunogenic composition comprising PRP and a polyanionic polymer to said one or more further antigens.

40. (canceled)

41. The method of claim 39, wherein the combination vaccine further comprises and adjuvant with a zero point charge greater than 8; wherein the polyanionic polymer prevents flocculation between the adjuvant and PRP and/or reduces the immunological interference that the adjuvant has on PRP.

42. The method of claim 39 wherein the immunogenic composition is added extemporaneously to said one or more further antigens.

43. The method of claim 39, wherein the immunogenic composition is lyophilised in the presence of a stabilizing excipient selected from the group consisting of: glucose, maltulose, iso-maltulose, lactulose, sucrose, sorbitol, maltose, lactose, iso-maltose, maltitol, lactitol, palatinit, trehalose, raffinose, stachyose, and melezitose.

44. The method of claim 39, wherein the immunogenic composition further comprises one or more conjugated meningococcal capsular oligosaccharides or polysaccharides selected from a group consisting of: MenC, MenY, MenA and MenW.

45. The method of claim 39, wherein the immunogenic composition further comprises one or more conjugated pneumococcal capsular oligosaccharides or polysaccharides.

46. The method of claim 39, wherein the adjuvant is aluminium hydroxide.

47. The method of claim 39, wherein the one or more further antigens comprise the following antigens: diphtheria toxoid, tetanus toxoid, pertussis toxoid, FHA and pertactin.

48. The method of claim 39, wherein the presence of the polyanionic polymer in the combination vaccine does not cause significant desorption of the one or more further antigens adsorbed to the adjuvant.

49-50. (canceled)

51. A kit comprising: i) a first immunogenic composition comprising a Haemophilus influenzae B capsular polysaccharide or oligosaccharide (PRP), and a polyanionic polymer; and ii) a second immunogenic composition comprising one or more antigens adsorbed onto an adjuvant with a zero point charge greater than 8.

52. (canceled)

53. The kit of claim 51, wherein the first immunogenic composition is lyophilised and further comprises a stabilizing excipient, and the second immunogenic composition is liquid.

54. The kit of claim 51, wherein the first immunogenic composition

further comprises one or more conjugated meningococcal capsular oligosaccharides or polysaccharides selected from a group consisting of: MenC, MenY, MenA and MenW.

55. The kit of claim 51, wherein the first immunogenic composition further comprises one or more conjugated pneumococcal capsular oligosaccharides or polysaccharides.

56. The kit of claim 51, wherein the adjuvant is aluminium hydroxide.

57. The kit of claim 51, wherein the second immunogenic composition comprises one or more antigens selected from a group consisting of: diphtheria toxoid, tetanus toxoid, pertussis toxoid, FHA and pertactin.

58. A method to prevent aggregation or flocculation of an immunogenic composition comprising addition of a polyanionic polymer to a saccharide antigen.

59. An immunogenic composition comprising a saccharide antigen with a pI less than 3, and a polyanionic polymer.

L3 ANSWER 3 OF 17 USPATFULL on STN
2006:68514 Novel vaccine.
Alchas, Paul G., Wayne, NJ, UNITED STATES
Garcon, Nathalie, Rixensart, BELGIUM
Slaoui, Moncef M, Hoverford, PA, UNITED STATES
Van Hoecke, Christian, Rixensart, BELGIUM
US 2006058736 A1 20060316
APPLICATION: US 2002-476331 A1 20020405 (10)
WO 2002-US10938 20020405 20040720 PCT 371 date
PRIORITY: US 2001-286821P 20010427 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CLM What is claimed is:
1. An intradermal delivery device for the intradermal delivery of a flu vaccine, the device comprising: i a container comprising a flu vaccine and having an outlet port; ii a needle in fluid communication with the outlet port, the needle having a forward end that is adapted to penetrate skin; and iii a limiter that surrounds the needle and has a skin engaging surface that is adapted to be received against the skin to receive an intradermal injection, the needle forward end extending beyond the skin engaging surface a selected distance such that the limiter portion limits an amount that the needle is able to penetrate through the skin.

2. The device of claim 1, wherein the drug container is a syringe including a generally hollow, cylindrical body portion and a plunger that is received within the reservoir, the plunger being selectively movable within the reservoir to cause the substance to be forced out of the outlet port during an injection.

3. The device of claim 1, including a hub portion that supports the needle and the hub portion is selectively secured to the drug container near the outlet port.

4. The device of claim 1, wherein the drug container is a syringe having a resevoir adapted to contain the vaccine, the syringe including a generally flat body portion that at least partially surrounds the reservoir, the body portion and the reservoir being made from two sheets of thermoplastic material such that side wails of the reservoir are selectively deflected toward each other to expel a substance from the reservoir during an injection.

5. The device of claim 4, including a hub that supports the needle and is selectively secured to the syringe near the outlet port and a receiver adjacent the outlet port that is generally circular and the hub is completely received within the receiver and wherein the limiter is integrally formed with the receiver such that the limiter is permanently supported by the body portion adjacent the outlet port.

6. The device of claim 5, wherein the skin engaging surface surrounds the needle, and has a thickness defined between an inner diameter and an outer diameter and wherein the inner diameter is at least five times greater than an outside diameter of the needle.

7. The device of claim 6, wherein the skin engaging surface is generally circular.

8. The device of claim 5, wherein the needle forward end extends away

from the hub in a first direction and a needle back end extends away from the hub in a second direction, and including a sealing membrane that closes off the outlet port and wherein the needle back end pierces the sealing membrane when the hub is received by the receiver.

9. The device of claim 4, including a hub that supports the needle and is selectively secured to the syringe near the outlet port and a receiver adjacent the outlet port that is generally circular and the hub is completely received within the receiver and wherein the limiter is formed separately from the receiver and is at least partially received by the receiver.

10. The device of claim 9, wherein the limiter and the hub are integrally formed into a single piece structure.

11. The device of claim 1, wherein the needle has a length and wherein the selected distance is much less than the needle length.

12. The device of claim 11, wherein the selected distance is fixed and is in the range from approximately 0.5 mm to approximately 3 mm.

13. The device of claim 1, wherein the skin engaging surface is generally flat and extends through a plane that is generally perpendicular to an axis of the needle.

14. The device of claim 1, wherein the skin engaging surface includes a central opening that is slightly larger than an outside dimension of the needle and the skin engaging surface is continuous.

15. The device of claim 1, wherein the skin engaging surface includes a contact surface area that is large enough to stabilise the assembly in a desired orientation relative to the skin.

16. The device of claim 1, wherein the desired orientation is generally perpendicular to the skin.

17. The device of claim 1, wherein the drug container is pre-filled with a substance.

18. A kit for use in intradermal flu vaccine delivery comprising: i a vaccine container comprising a flu vaccine and ii a hypodermic needle assembly, the assembly comprising: a hub portion that is able to be attached to a drug container; a needle supported by the hub portion, the needle having a hollow body with a forward end extending away from the hub portion; and a limiter portion that surrounds the needle and extends away from the hub portion toward the forward end of the needle, the limiter portion having a skin engaging surface that is adapted to be received against the skin of an animal to receive an intradermal injection, the needle forward end extending beyond the skin engaging surface a selected distance such that the limiter portion limits an amount that the needle is able to penetrate through the skin of an animal.

19. The kit according to claim 18, wherein the hub portion and the limiter portion are integrally formed as a single piece made from a plastic material.

20. The kit according to claim 18, wherein wherein the hub portion and the limiter portion are formed as separate pieces.

21. The kit according to claim 20, wherein the limiter portion includes an inner cavity that receives at least a portion of the hub portion and the inner cavity includes an abutment surface that engages corresponding structure on the hub portion to thereby limit the amount that the needle forward end extends beyond the skin engaging surface.

22. The kit according to claim 20, wherein the limiter portion is integrally formed as part of the syringe and the hub portion is received within the limiter portion.

23. The kit according to claim 22, wherein the skin engaging surface surrounds the needle, and has a thickness defined between an inner diameter and an outer diameter and wherein the inner diameter is at least five times greater than an outside diameter of the needle.

24. The kit according to claim 23, wherein the skin engaging surface is generally circular.

25. The kit according to claim 18, wherein the skin engaging surface

includes a central opening that is slightly larger than an outside diameter of the needle and the skin engaging surface is continuous.

26. The kit according to claim 18, wherein the skin engaging surface is generally flat and extends through a plane that is generally perpendicular to an axis of the needle.

27. The kit according to claim 18, wherein the selected distance that the forward end of the needle extends beyond the skin engaging surface is fixed.

28. The kit according to claim 18, wherein the selected distance is in the range from approximately 0.5 mm to approximately 3 mm.

29. The kit according to claim 18, wherein the skin engaging surface includes a contact surface area that is large enough to stabilise the assembly in a desired orientation relative to the skin.

30. The kit according to claim 29, wherein the desired orientation is generally perpendicular to the skin.

31. The kit according to claim 18, wherein the drug container is a syringe and the animal is human.

32. A device according to any of claims, or a kit according to any of claims 1-31, wherein the flu vaccine is obtainable by the following process: (i) harvesting of virus-containing material from a culture; (ii) clarification of the harvested material to remove non-virus material; (iii) concentration of the harvested virus; (iv) a further step to separate whole virus from non-virus material; (v) splitting of the whole virus using a suitable splitting agent in a density gradient centrifugation step; (vi) filtration to remove undesired materials; wherein the steps are performed in that order but not necessarily consecutively.

33. A device or kit according to claim 32, wherein the intradermal flu vaccine is a trivalent non-live vaccine.

34. A device or kit according to claim 32, wherein the virus is grown on embryonated hen eggs and the harvested material is allantoic fluid.

35. A device or kit according to claim 32, wherein the clarification step is performed by centrifugation at a moderate speed.

36. A device or kit according to claim 32, wherein the concentration step employs an adsorption method such as $CaHPO_4$ adsorption.

37. A device or kit according to claim 32, wherein the further separation step (iv) is a zonal centrifugation separation using a sucrose gradient.

38. A device or kit according to claim 32, wherein the splitting step is performed in a further sucrose gradient, wherein the sucrose gradient contains the splitting agent.

39. A device or kit according to claim 38, wherein the splitting agent is sodium deoxycholate.

40. A device or kit according to claim 32, wherein the filtration step (vi) is an ultrafiltration step which concentrates the split virus material.

41. A device or kit according to claim 32, wherein there is at least one sterile filtration step, optionally at the end of the process.

42. A device or kit according to claim 32, wherein an inactivation step is performed prior to the final filtration step.

43. A device or kit according to claim 32, wherein the method comprises the further step of adjusting the concentration of one or more detergents in the vaccine composition.

44. A device or kit according to claim 32, wherein the vaccine is provided in a dose volume of between about 0.1 and about 0.2 ml.

45. A device or kit according to claim 32, wherein the vaccine is provided with an antigen dose of 1-5 µg haemagglutinin per strain of influenza present.

46. A device or kit according to claim 32, wherein the vaccine meets the EU criteria for at least two strains.

47. A device or kit according to claim 32, wherein the vaccine further comprises a bile acid or cholic acid, or derivative thereof such as sodium deoxycholate.

48. A device or kit according to claim 32, wherein the vaccine comprises at least one non-ionic surfactant.

49. A device or kit according to claim 32, wherein the at least one non-ionic surfactant selected from the group consisting of the octyl- or nonylphenoxy polyoxyethanols (for example the commercially available Triton.TM. series), polyoxyethylene sorbitan esters (Tween.TM. series) and polyoxyethylene ethers or esters of general formula (I): $HO(CH_{2CH2O})_{n-A-R}$ (I) wherein n is 1-50, A is a bond or --(O)--, R is C_{1-50} alkyl or phenyl C_{1-50} alkyl; and combinations of two or more of these.

50. A device or kit according to claim 49, wherein the vaccine comprises a combination of polyoxyethylene sorbitan monooleate (Tween 80) and t-octylphenoxy polyethoxyethanol (Triton X-100).

L3 ANSWER 4 OF 17 USPATFULL on STN
2005:18867 Vaccines containing a saponin and a sterol.
Garcon, Nathalie Marie-Josephe Claude, Wavre, BELGIUM
Friede, Martin, Brussels, BELGIUM
SmithKline Beecham Biologicals s.a., Rixensart, BELGIUM (non-U.S. corporation)
US 6846489 B1 20050125
APPLICATION: US 2000-478705 20000106 (9)
PRIORITY: GB 1995-8326 19950425
GB 1995-13107 19950628
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. An adjuvant composition comprising a sterol and a substantially pure preparation of QS21, characterized in that the adjuvant composition is in the form of an ISCOM.

2. An adjuvant composition according to claim 1, wherein the QS21 is at least 90% pure.

3. An adjuvant composition according to claim 1, wherein the QS21 is at least 95% pure.

4. An adjuvant composition according to claim 1, wherein the QS21 is at least 98% pure.

L3 ANSWER 5 OF 17 USPATFULL on STN
2004:126484 Novel vaccine.
Garcon, Nathalie, Rixensart, BELGIUM
Slaoui, Moncef Mohamed, Rixensart, BELGIUM
Van Hoecke, Christian, Rixensart, BELGIUM
US 2004096463 A1 20040520
APPLICATION: US 2004-469087 A1 20040107 (10)
WO 2002-EP1843 20020221
PRIORITY: GB 2001-4542 20010223
GB 2001-8366 20010403
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. The use of an influenza antigen preparation obtainable by the following process, in the manufacture of an intradermal flu vaccine: (i) harvesting of virus-containing material from a culture; (ii) clarification of the harvested material to remove non-virus material; (iii) concentration of the harvested virus; (iv) a further step to separate whole virus from non-virus material; (v) splitting of the whole virus using a suitable splitting agent in a density gradient centrifugation step; (vi) filtration to remove undesired materials; wherein the steps are performed in that order but not necessarily consecutively.

2. The use according to claim 1 wherein the intradermal flu vaccine is a trivalent vaccine.

3 The use according to claim 1 or claim 2 wherein the virus is grown on

embryonated hen eggs and the harvested material is allantoic fluid.

4. The use according to any one of claims 1 to 3 wherein the clarification step is performed by centrifugation at a moderate speed.

5. The use according to any one of claims 1 to 4 wherein the concentration step employs an adsorption method such as $CaHPO_4$ adsorption.

6. The use according to any one of claims 1 to 5 wherein the further separation step (iv) is a zonal centrifugation separation using a sucrose gradient.

7. The use according to claim 6 wherein the splitting step is performed in a further sucrose gradient, wherein the sucrose gradient contains the splitting agent.

8. The use according to claim 7 wherein the splitting agent is sodium deoxycholate.

9. The use according to any one of claims 1 to 8 wherein the filtration step (vi) is an ultrafiltration step which concentrates the split virus material.

10. The use according to any one of claims 1 to 9 wherein there is at least one sterile filtration step, optionally at the end of the process.

11. The use according to any one of claims 1 to 10 wherein an inactivation step is performed prior to the final filtration step.

12. The use according to any one of claim 1 to II wherein the method comprises the further step of adjusting the concentration of one or more detergents in the vaccine composition.

13. The use according to any one of claims 1 to 12 wherein the vaccine is provided in a dose volume of between about 0.1 and about 0.2 ml.

14. The use according to any one of claims 1 to 13 wherein the vaccine is provided with an antigen dose of 1-7.5 µg haemagglutinin per strain of influenza present.

15. The use according to any one of claims 1 to 14 wherein the vaccine further comprises an adjuvant such as an adjuvant comprising a combination of cholesterol, a saponin and an LPS derivative.

16. The use of a trivalent, split influenza antigen preparation in the manufacture of a vaccine for intradermal delivery.

17. The use according to claim 16 wherein the intradermal vaccine comprises at least one non-ionic surfactant.

18. A pharmaceutical kit comprising an intradermal delivery device and an influenza vaccine obtainable by the following process: (i) harvesting of virus-containing material from a culture; (ii) clarification of the harvested material to remove non-virus material; (iii) concentration of the harvested virus; (iv) a further step to separate whole virus from non-virus material; (v) splitting of the whole virus using a suitable splitting agent in a density gradient centrifugation step; (vi) filtration to remove undesired materials; wherein the steps are performed in that order but not necessarily consecutively.

19. The pharmaceutical kit according to claim 18 wherein the intradermal delivery device is a short needle delivery device.

L3 ANSWER 6 OF 17 USPATFULL on STN
2004:94282 Novel vaccine.
Garcon, Nathalie, Rixensart, BELGIUM
Slaoui, Moncef Mohamed, Rixensart, BELGIUM
Van Hoecke, Christian, Rixensart, BELGIUM
US 2004071734 A1 20040415
APPLICATION: US 2003-469191 A1 20030825 (10)
WO 2002-EP1844 20020221
PRIORITY: GB 2001-45384 20010223
GB 2001-75118 20010326
GB 2001-83658 20010403
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. The use of a trivalent, non-live influenza antigen preparation in the manufacture of a one-dose influenza vaccine for intradermal delivery.

2. The use according to claim 1 wherein the antigen preparation is a split influenza preparation.

3. The use according to claim 1 or claim 2 wherein the influenza antigen is egg-derived.

4. The use according to any one of claims 1 to 3 wherein the vaccine meets the EU criteria for at least two strains.

5. The use according to any one of claims 1 to 4 wherein the vaccine comprises at least one non-ionic surfactant selected from the group consisting of the octyl- or nonylphenoxy polyoxyethanols (for example the commercially available Triton.TM.series), polyoxyethylene sorbitan esters (Tween series) and polyoxyethylene ethers or esters of general formula (I): $HO(CH_2CH_2O)_{n-A-R}$ (I) wherein n is 1-50, A is a bond or --C(O)--, R is C_{1-50} alkyl or phenyl C_{1-50} alkyl; and combinations of two or more of these.

6. The use according to claim 5 wherein the vaccine comprises a combination of polyoxyethylene sorbitan monooleate (Tween 80) and t-octylphenoxy polyethoxyethanol (Triton X-100).

7. The use according to any one of claims 1 to 6 wherein the vaccine farther comprises a bile acid or cholic acid, or derivative thereof such as sodium deoxycholate.

8. The use according to any one of claims 1 to 7 wherein the vaccine is provided in a dose volume of between about 0.1 and about 0.2 ml.

9. The use according to any one of claims 1 to 8 wherein the vaccine is provided with an antigen dose of 1-7.5 µg haemagglutinin per strain of influenza present.

10. The use according to any one of claims 1 to 9 wherein the vaccine further comprises an adjuvant such as an adjuvant comprising a combination of cholesterol, a saponin and an LPS derivative.

11. The use according to any one of claims 1 to 10 wherein the vaccine is provided in an intradermal delivery device.

12. The use according to claim 11 wherein the device is a short needle delivery device.

13. The use of an influenza antigen preparation obtainable by the following process, in the manufacture of an intradermal flu vaccine: (i) harvesting of virus-containing material from a culture; (ii) clarification of the harvested material to remove non-virus material; (iii) concentration of the harvested virus; (iv) a further step to separate whole virus from non-virus material; (v) splitting of the whole virus using a suitable splitting agent in a density gradient centrifugation step; (vi) filtration to remove undesired materials; wherein the steps are performed in that order but not necessarily consecutively.

14. A pharmaceutical kit comprising an intradermal delivery device and a trivalent non-live influenza vaccine.

15. The pharmaceutical kit according to claim 14 wherein the intradermal delivery device is a short needle device.

16. The pharmaceutical kit according to claim 14 or claim 15 wherein the volume of vaccine is between about 0.05 and 0.2 ml.

L3 ANSWER 7 OF 17 USPATFULL on STN
2004:57036 Vaccines.
Momin, Patricia Marie, Brussels, BELGIUM
Garcon, Nathalie Marie-Josephe, Wavre, BELGIUM
SmithKline Beecham Biologicals S.A. (non-U.S. corporation)
US 2004043038 A1 20040304
APPLICATION: US 2003-654279 A1 20030903 (10)
PRIORITY: GB 1993-26253 19931223
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:

1. A vaccine composition comprising an antigen and/or antigenic composition, QS21, 3 De-O-acylated monophosphoryl lipid A (3D-MPL) and an oil in water emulsion wherein the oil in water emulsion has the following composition: a metabolisible oil, such as squalene, alpha tocopherol and tween 80.

2. A vaccine as claimed in claim 1 wherein the ratio of QS21:3D-MPL is from 1:10 to 10:1.

3. A vaccine composition as claimed in claim 1 or 2 capable of invoking a cytolytic T cell response in a mammal to the antigen or antigenic composition.

4. A vaccine composition as claimed in any of claims 1 to 3 capable of stimulating interferon γ production.

5. A vaccine composition as claimed in any of claims 1 to 4 wherein the ratio of QS21:3D-MPL is from 1:1 to 1:2.5.

6. A vaccine composition as claimed herein comprising an antigen or antigenic composition derived from any of Human Immunodeficiency Virus, Feline Immunodeficiency Virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A,B,C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium or Toxoplasma.

7. A vaccine as claimed in any of claim 1 to 5 wherein the antigen is a tumour antigen.

8. Use of composition as defined in any of claims 1 to 5 for the manufacture of a vaccine for the prophylatic treatment of viral, bacterial, or parasitic infections.

9. Use of composition as defined in any of claims 1 to 5 for the manufacture of a vaccine for the immunotherapeutic treatment of viral, bacterial, parasitic infections or cancer.

10. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition according to any of claims 1 to 5.

11. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition according to any of claims 1 to 5.

12. A process for making a vaccine composition according to claims 1 to 5 comprising admixing QS21, 3D-MPL and the oil in water emulsion as defined in claim 1 with an antigen or antigenic composition.

13. A vaccine composition comprising an antigen or antigenic composition in association with an oil in water emulsion which emulsion comprises: a metabolisable oil, alpha tocopherol, and tween 80.

L3 ANSWER 8 OF 17 USPATFULL on STN
2003:253451 Vaccines.
Momin, Patricia Marie, Brussels, BELGIUM
Garcon, Nathalie Marie-Josephe, Wavre, BELGIUM
SmithKline Beecham Biologicals s.a., Rixensart, BELGIUM (non-U.S. corporation)
US 6623739 B1 20030923
APPLICATION: US 2000-513255 20000224 (9)
PRIORITY: GB 1993-26253 19931223
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. An immunogenic composition comprising an antigen and/or antigen composition and an adjuvant consisting of a metabolizable oil and alpha tocopherol in the form of an oil in water emulsion.

2. The immunogenic composition according to claim 1 wherein said metabolizable oil is squalene.

3. The immunogenic composition according to claim 1 wherein said antigen and/or antigenic composition is prepared from the group consisting of Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, HepatitisA , B, C, or E, Respiratory Syncytial Virus, Human Papilloma Virus, Influenza Virus, Salmonella, Neisseria, Borrelia,

Chlamydia, Bordetella, Plasmodium and Toxoplasma, Feline Immunodeficiency Virus, and Human Immunodeficiency Virus.

4. The immunogenic composition according to claim 1 wherein the antigen or antigen composition is a tumor antigen.

5. A process for making an immunogenic composition according to claim 1 comprising admixing the oil and water emulsion with an antigen or antigen composition.

6. An immunogenic composition according to claim 1 wherein said antigen and/or antigenic composition is prepared from the group consisting of Human Immunodeficiency Virus and Feline Immunodeficiency Virus.

7. A vaccine composition comprising an antigen and/or antigen composition and an adjuvant consisting of a metabolizable oil and alpha tocopherol in the form of an oil in water emulsion.

8. The vaccine composition of claim 7 further comprising TWEEN 80 (polyoxyethelene sorbitan monooleate).

9. The vaccine composition of claim 7 further comprising a stabilizer.

10. The vaccine composition of claim 7 wherein the ratio of metablizable oil: alpha tocopherol is equal or less than 1.

11. The immunogenic composition of claim 1 further comprising TWEEN 80 (polyoxyethelene sorbitan monooleate).

12. The immunogenic composition of claim 1 further comprising a stabilizer.

13. The immunogenic composition of claim 1 wherein the ratio of metablizable oil: alpha tocopherol is equal or less than 1.

14. The vaccine composition according to claim 7 wherein said metabolizable oil is squalene.

15. A process for making a vaccine according to claim 7 comprising admixing the oil and water emulsion with an antigen or antigen composition.

L3 ANSWER 9 OF 17 USPATFULL on STN
2003:140134 Oil in water emulsions containing saponins.
Garcon, Nathalie, Wavre, BELGIUM
Momin, Patricia Marie Christine Aline Francoise, Brussells, BELGIUM
SmithKline Beecham Biologicals S.A. (non-U.S. corporation)
US 2003095974 A1 20030522
APPLICATION: US 2002-139815 A1 20020506 (10)
PRIORITY: GB 1997-18902 19970905
GB 1997-20982 19971002
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. A composition comprising an oil in water emulsion and a saponin, wherein said oil is a metabolisable oil, characterised in that the ratio of the metabolisable oil:saponin (w/w) is in the range of 1:1 to 200:1.

2. A composition comprising an oil in water emulsion and a saponin, wherein said oil is a metabolisable oil, characterised in that the ratio of the metabolisable oil:saponin (w/w) is in the range of 1:1 to 100:1.

3. A composition comprising an oil in water emulsion and a saponin, wherein said oil is a metabolisable oil, characterised in that the ratio of the metabolisable oil:saponin (w/w) is substantially 48:1.

4. A composition as claimed in any of claims 1 to 3, wherein the saponin is QuilA or derivative thereof, such as QS21.

5. A composition as claimed in any of claims 1 to 4, where the metabolisable oil is squalene.

6. A composition as claimed in any of claims 1 to 5, further comprising a sterol.

7. A composition as claimed in claim 6, where the sterol is cholesterol.

8. A composition as claimed in claims 1 to 7, further comprising one or

more other immunomodulators.

9. A composition as claimed in any of the preceding claims, further comprising one or more other immunomodulators, which immunomodulator is selected from the group comprising: 3D-MPL, α-tocopherol.

10. A composition as claimed in claim 9, wherein the ratio of QS21:3D-MPL (w/w) is from 1:10 to 10:1.

11. A composition as claimed in claim 9 or 10, wherein the ratio of QS21:3D-MPL (w/w) is from 1:1 to 1:2.5.

12. A composition as claimed in claim 7, wherein the ratio of QS21:cholesterol (w/w) is in the range of 1:1 to 1:20.

13. A vaccine composition comprising a composition as claimed in any of claims 1 to 12, further comprising an antigen or antigenic preparation.

14. A vaccine composition as claimed in claim 13, where the antigen or antigenic preparation is prepared from the group comprising: Human Immunodeficiency Virus; Herpes Simplex Virus type 1; Herpes Simplex Virus type 2; Human Cytomegalovirus; Hepatitis A, B, C or E; Respiratory Syncitial Virus, Human Papilloma Virus; Influenza Virus; Salmonella; Neisseria; Borrelia; Chlamydia; Bordetella; TB; EBV; Plasmodium and Toxoplasma.

15. A vaccine composition as claimed in claim 13, wherein the antigen or antigenic preparation is a combination of the Malaria antigens RTS,S and TRAP.

16. A vaccine composition as claimed in claim 13, where the antigen or antigenic preparation is, or is derived from, a tumour or host derived antigen.

17. A composition as claimed herein, wherein the oil in water emulsion comprises oil droplets which have a diameter which is less that 1 micron.

18. A composition as claimed herein, wherein the oil in water emulsion comprises oil droplets which are in the range of 120 to 750 nm in diameter.

19. A composition as claimed herein, wherein the oil in water emulsion comprises oil droplets which are in the range of 120 to 600 nm in diameter.

20. A vaccine composition as claimed herein which is capable of invoking a cytolytic T-cell response in a mammal to the antigen or antigenic composition.

21. A vaccine composition as claimed herein which is capable of stimulating interferon-γ production in a mammal to the antigen or antigenic composition.

22. A vaccine adjuvant composition as claimed in any of the preceding claims for use in medicine.

23. A method for manufacturing a vaccine as claimed in any one of claims 13 to 16, comprising admixing an oil in water emulsion; QS21; cholesterol; 3D-MPL; α-tocopherol; and an antigen or antigenic preparation.

23. The use of a composition as substantially herein described in the manufacture of a vaccine suitable for the treatment of a human susceptible to or suffering from a disease.

24. The treatment of an individual susceptible to or suffering from a disease by the administration of a composition as substantially herein described.

25. The treatment of an individual susceptible to or suffering from a disease by the administration of a vaccine as substantially herein described.

26. A method of stabilising a saponin present in a composition of claim 1, comprising the addition of a sterol into the oil phase of said oil in water emulsion.

27. A method as claimed in claim 26, wherein the saponin is QS21.

28. A method as claimed in claims 26 or 27, wherein the sterol is cholesterol.

L3 ANSWER 10 OF 17 USPATFULL on STN
2003:30357 Vaccine composition.
Artois, Claude, Rhode-St-Genese, BELGIUM
Heyder, Koen De, Grimbergen, BELGIUM
Desmons, Pierre, Nivelles, BELGIUM
Garcon, Nathalie, Wavre, BELGIUM
Mainil, Roland, Ghlin, BELGIUM
SmithKline Beecham Biologicals, s.a. (non-U.S. corporation)
US 2003022304 A1 20030130
APPLICATION: US 2002-217572 A1 20020813 (10)
PRIORITY: GB 1998-6456 19980325
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:

1. A method to reduce interference of a capsular polysaccharide component of a conjugated Haemophilus influenzae B vaccine (Hib) in a combination vaccine comprising DTPa, wherein such method comprises: (i) selecting one or more antigen(s) to be adsorbed onto aluminium hydroxide adjuvant; (ii) pre-saturating the aluminium hydroxide adjuvant with the selected antigens; (iii) selecting Hib plus one or more additional antigen(s) to be adsorbed onto aluminium phosphate; (iv) adsorbing Hib and said additional antigens onto aluminium phosphate; (v) combining all antigens in said vaccine.

2. The method of claim 1 wherein the Hib adsorbed onto aluminium phosphate is mixed extemporaneously with the other antigens of the combination vaccine.

3. The method of claims 1 to 2 wherein the combination vaccine additionally comprises one or more antigens selected from the group: Hepatitis B surface antigen (HBsAg), inactivated Hepatitis A virus, inactivated Polio virus, N. meningitidis A capsular polysaccharide, N. meningitidis C capsular polysaccharide, Streptococcus pneumoniae capsular polysaccharide, Streptococcus pneumoniae proteins, Moraxella catarrhalis outer membrane proteins, non-typeable Haemophilus influenzae outer membrane proteins, N. meningitidis B outer membrane proteins.

4. The method of claim 3 wherein the combination vaccine comprises Hepatitis B surface antigen adsorbed onto aluminium phosphate.

5. The method of any one of claims 1 to 4 wherein the ratio of aluminium phosphate to aluminium hydroxide adjuvant present in the combination vaccine ranges from 1:1 to 20:1.

6. The method of any one of claims 1 to 5 wherein all the antigens of the combination vaccine are adsorbed onto aluminium phosphate, with the proviso that pertactin is adsorbed onto aluminium hydroxide.

7. The method of claim 6 wherein the pertactin adsorbed onto aluminium hydroxide is combined with unadsorbed inactivated Polio virus antigens before being combined with the aluminium phosphate adsorbed antigens.

8. The method of any one of claims 1 to 5 wherein all the antigens of the combination vaccine are adsorbed onto aluminium hydroxide, with the proviso that HBsAg and Hib are adsorbed onto aluminium phosphate.

9. The method of claim 8 wherein the antigens adsorbed onto aluminium hydroxide are combined before the HBsAg antigen, adsorbed onto aluminium phosphate, is added and Hib, adsorbed onto aluminium phosphate, is combined after the HBsAg antigen has been added.

10. The method of claims 8 to 9 wherein additional free aluminium phosphate is added to the combination vaccine.

11. A method to reduce interference of a capsular polysaccharide component of a conjugated Haemophilus influenzae B vaccine (Hib) in a combination vaccine comprising DTPa, wherein such method comprises: (i) selecting one or more antigen(s) to be adsorbed onto aluminium hydroxide adjuvant; (ii) pre-saturating the aluminium hydroxide adjuvant with the selected antigens; (iii) selecting one or more additional antigen(s) to be adsorbed onto aluminium phosphate; (iv) adsorbing said additional antigens onto aluminium phosphate; (vi) combining all antigens in said vaccine with unadjuvanted Hib.

12. A method to reduce interference of a capsular polysaccharide component of a conjugated Haemophilus influenzae B vaccine (Hib) in a combination vaccine comprising DTPa, wherein such method comprises: (i) selecting one or more antigen(s) to be adsorbed onto aluminium hydroxide adjuvant; (ii) pre-saturating the aluminium hydroxide adjuvant with the selected antigens; (iii) selecting one or more additional antigen(s) to be adsorbed onto aluminium phosphate; (iv) adsorbing said additional antigens onto aluminium phosphate; (v) combining all antigens in said vaccine; (vi) extemporaneously adding either unadjuvanted Hib or Hib adsorbed onto aluminium phosphate.

13. The method of claim 12 wherein the combination vaccine additionally comprises one or more antigens selected from the group: Hepatitis B surface antigen (HBsAg), inactivated Hepatitis A virus, inactivated Polio virus, N. meningitidis A capsular polysaccharide, N. meningitidis C capsular polysaccharide, Streptococcus pneumoniae capsular polysaccharide, Streptococcus pneumoniae proteins, Moraxella catarrhalis outer membrane proteins, non-typeable Haemophilus influenzae outer membrane proteins, N. meningitidis B outer membrane proteins.

14. The method of claim 13 wherein the combination vaccine comprises Hepatitis B surface antigen adsorbed onto aluminium phosphate.

15. The method of any one of claims 12 to 14 wherein the ratio of aluminium phosphate to aluminium hydroxide adjuvant present in the combination vaccine ranges from 1:1 to 20:1.

16. The method of any one of claims 12 to 15 wherein all the antigens of the combination vaccine are adsorbed onto aluminium phosphate, with the proviso that pertactin is adsorbed onto aluminium hydroxide and that Hib is added either unadjuvanted or adsorbed onto aluminium phosphate.

17. The method of claim 16 wherein the pertactin adsorbed onto aluminium hydroxide is combined with unadsorbed inactivated Polio virus antigens before being combined with the aluminium phosphate adsorbed antigens.

18. The method of any one of claims 12 to 15 wherein all the antigens of the combination vaccine are adsorbed onto aluminium phosphate, with the proviso that pertactin, diphtheria toxoid, pertussis toxoid, filamentous haemagglutinin are adsorbed onto aluminium hydroxide and that Hib is added either unadjuvanted or adsorbed onto aluminium phosphate.

19. The method of claim 18 wherein the pertactin, diphtheria toxoid, pertussis toxoid, filamentous haemagglutinin adsorbed onto aluminium hydroxide are combined with unadsorbed inactivated Polio virus antigens before being combined with the aluminium phosphate adsorbed antigens.

20. The method of any one of claims 12 to 15 wherein all the antigens of the combination vaccine are adsorbed onto aluminium hydroxide, with the proviso that HBsAg is adsorbed onto aluminium phosphate and that Hib is added either unadjuvanted or adsorbed onto aluminium phosphate.

21. The method of claim 20 wherein the antigens adsorbed onto aluminium hydroxide are combined before the HBsAg antigen, adsorbed onto aluminium phosphate, is added.

22. The method of claims 20 to 21 wherein additional free aluminium phosphate is added to the combination vaccine before the extemporaneous addition of unadjuvanted Hib.

23. The method of any one of claims 12 to 22 wherein one or more of the antigens selected to pre-saturate the aluminium hydroxide has been previously adsorbed onto aluminium phosphate.

24. The method of claim 23 wherein diphtheria toxoid adsorbed onto aluminium phosphate is one of the antigens selected to pre-saturate the aluminium hydroxide.

25. The method of any one of claims 1 to 24 wherein the combination vaccine is buffered with L-histidine.

26. A combination vaccine obtained by the method of any one of claims 1 to 25.

27. A method of vaccinating against diphtheria, tetanus, pertussis, and H. influenzae type b comprising administering a pharmaceutically effective amount of the combination vaccine of claim 26.

L3 ANSWER 11 OF 17 USPATFULL on STN
2003:13075 Vaccine comprising an iscom consisting of sterol and saponin which is free of additional detergent.
Friede, Martin, Farnham, UNITED KINGDOM
Garcon, Nathalie, Wavre, BELGIUM
SmithKline Beecham Biologicals, S.A., Rixensart, BELGIUM (non-U.S. corporation)
US 6506386 B1 20030114
WO 2000007621 20000217
APPLICATION: US 2001-744800 20010604 (9)
WO 1999-EP5587 19990803
PRIORITY: GB 1998-17052 19980805
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:

1. An adjuvant composition comprising a sterol, a saponin, and a phospholipid, characterised in that the adjuvant is in the form of an ISCOM and that it is free of additional detergent, other than the saponin.

2. An adjuvant composition as claimed in claim 1, wherein the ratio of saponin:sterol (w/w) exceeds 1.

3. An adjuvant composition as claimed in claim 1, wherein the ratio of saponin to sterol is in the range of 1:1 to 100:1 (w/w).

4. An adjuvant composition as claimed in claim 1, wherein the ratio of saponin to sterol is 5:1.

5. An adjuvant composition as claimed in any one of claims 1 to 4, wherein the saponin is Quil A or extract thereof.

6. An adjuvant composition as claimed in claim 5, wherein the extract of Quil A is QS21.

7. An adjuvant composition as claimed in claim 1, wherein the sterol is cholesterol.

8. An adjuvant composition as claimed in claim 1, wherein the phospholipid is phosphatidylcholine.

9. An adjuvant composition as claimed in claim 8, wherein phosphatidylcholine is dioloeoylphosphatidylcholine or dilauryl phosphatidylcholine.

10. An adjuvant composition as claimed in claim 7, wherein the ratio of cholesterol to phospholipid is 50% (w/w).

11. An adjuvant composition as claimed in claim 10, wherein the ratio of cholesterol to phospholipid is 20-25% (w/w).

12. A vaccine comprising an adjuvant composition as claimed in any one of claims 1 to 11, further comprising an antigen.

13. A vaccine composition as claimed in claim 12, wherein the antigen is an antigen or antigenic composition derived from any of Human Immunodeficiency Virus, Feline Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C, or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium, or Toxoplasma.

14. A process for the manufacture of an adjuvant composition, comprising the following steps: (a) the formation of cholesterol containing small unilamellar liposomes (SUL) in the absence of detergent; and (b) admixing the preformed liposomes with saponin at a ratio of saponin:cholesterol (w/w) exceeding 1.

15. A process for the manufacture of a vaccine composition, comprising the following steps: (a) taking an adjuvant composition produced according to the process of claim 14; and (b) adding an antigen or an antigenic composition.

L3 ANSWER 12 OF 17 USPATFULL on STN
2002:317152 Hepatitis B vaccine.
Hauser, Pierre, Chaumont Gistoux, BELGIUM
Garcon, Nathalie Marie-Josephe Claude, Wavre, BELGIUM

Desmons, Pierre, Nivelles, BELGIUM
SmithKline Beecham Biologicals S.A., Rixensart, BELGIUM (non-U.S. corporation)
US 6488934 B1 20021203
APPLICATION: US 2000-730930 20001206 (9)
PRIORITY: GB 1995-3863 19950225
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:

1. A vaccine composition comprising a hepatitis B antigen, 3-O-deacylated monophosphoryl lipid A and aluminum phosphate and further comprising a component selected from the group consisting of: a killed attenuated hepatitis A virus, diphtheria antigen, tetanus antigen, pertussis antigen, Haemophilus influenzae b (Hib) antigen, polio antigen, meningitidis A antigen, meningitidis B antigen, meningitidis C antigen and combinations thereof.

2. A vaccine composition according to claim 1 further comprising a component selected from the group consisting of: aDTP (diptheria-tetanus-pertussis)-HBsAg combination, an Hib-HBsAg combination, a DTP-Hib-HBsAg combination and an IPV (inactivated polio vaccine)-DTP-Hib-HBsAg combination, and additionally comprising a killed attenuated hepatitis A virus.

3. A combination vaccine comprising a hepatitis B antigen, 3-O-deacylated monophosphoryl lipid A, aluminum phosphate and one or more components selected from the group consisting of: a killed attenuated hepatitis A virus, diphtheria antigen, tetanus antigen, pertussis antigen, Haemophilus influenzae b (Hib) antigen, polio antigen, meningitidis A antigen, meningitidis B antigen, and meningitidis C antigen.

4. A method of inducing neutralizing antibody titres in a human susceptible to or suffering from an infection by administering the vaccine combination as claimed in claim 1.

5. A method of preventing infections in humans which comprises administering an effective amount of the vaccine composition as claimed in claim 1.

6. A method of treating a human subject suffering from an ongoing infection comprising administrating an effective amount of a therapeutic vaccine composition as claimed in claim 1.

7. A method of inducing neutralizing antibody titres in a human susceptible to or suffering from an infection by administering the vaccine combination as claimed in claim 3.

8. A method of preventing infections in humans which comprises administering an effective amount of the vaccine composition as claimed in claim 3.

9. A method of treating a human subject suffering from an ongoing infection comprising administrating an effective amount of a therapeutic vaccine composition as claimed in claim 3.

10. A method of inducing neutralizing antibody titres in a human susceptible to or suffering from an infection by administering the vaccine combination as claimed in claim 2.

11. A method of preventing infections in humans which comprises administering an effective amount of the vaccine composition as claimed in claim 2.

12. A method of treating a human subject suffering from an ongoing infection comprising administrating an effective amount of a therapeutic vaccine composition as claimed in claim 2.

13. A vaccine composition according to claim 1 wherein the killed attenuated hepatitis A virus is obtained from a HM- 175 strain of Hepatitis A virus.

14. A vaccine composition according to claim 2 wherein the killed attenuated hepatitis A virus is obtained from a HM-175 strain of Hepatitis A virus.

15. A vaccine composition according to claim 3 wherein the killed attenuated hepatitis A virus is obtained from a HM-175 strain of Hepatitis A virus.

L3 ANSWER 13 OF 17 USPATFULL on STN
2002:81034 Vaccines.
Garcon, Nathalie, Wavre, BELGIUM
Momin, Patricia Marie Christine Aline Francoise, Brussells, BELGIUM
SmithKline Beecham Biologicals, s.a., Rixensart, BELGIUM (non-U.S. corporation)
US 6372227 B1 20020416
WO 9912565 19990318
APPLICATION: US 2000-486996 20000424 (9)
WO 1998-EP5714 19980902 20000424 PCT 371 date
PRIORITY: GB 1997-18901 19970905
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:

1. A composition comprising an oil in water emulsion having an oil phase and an aqueous phase and a saponin, wherein the oil phase of said oil in water emulsion comprises a metabolizable oil and a sterol and the saponin is in the aqueous phase.

2. A composition as claimed in claim 1, where the sterol is cholesterol.

3. A composition as claimed in claim 1, wherein said metabolizable oil is squalene.

4. A composition as claimed in claim 1, wherein said saponin is a derivate of QuliA.

5. A composition as claimed in claim 4, wherein said QuilA derivative is selected from the group consisting of QS21 and QS17.

6. A composition as claimed in claim 1, further containing one or more other immunomodulators.

7. A composition as claimed in claim 6, wherein the immunomodulators are selected from the group consisting of 3D-MPL and α-tocopherol.

8. A composition for raising an immune response comprising a composition as claimed in any one of claims 1 to 7, further comprising an antigen or antigenic preparation.

9. A composition for raising an immune response as claimed in claim 8, where the antigen or antigenic preparation is prepared from the group comprising: Human Immunodeficiency Virus; Herpes Simplex Virus type 1; Herpes Simplex Virus type 2, Human Cytomegalovirus; Hepatitis A, B, C or E; Respiratory Syncytial Virus, Human Papilloma Virus; Influenza Virus, Salmonella; Neisseria,; Borrelia; Chlamydia; Bordetella; Plasmodium, Toxoplasma, tuberculosis and EBV.

10. A composition for raising an immune response as claimed in claim 8, wherein the antigen or antigenic preparation is a combination of the Malaria antigens RTS, S and TRAP.

11. A composition for raising an immune response as claimed in claim 8, wherein the antigen or antigenic preparation is, or is derived from, a tumor or host derived antigen.

12. A method for manufacturing a composition as claimed in claim 8 comprising admixing (a) an oil in water emulsion wherein the oil droplets comprise a sterol; (b) an aqueous solution of QS21; and (c) an antigen or antigenic preparation.

13. A method for manufacturing a composition as claimed in claim 12 wherein said sterol is cholesterol.

14. A method of treating an individual susceptible to or suffering from a disease by the administration of a vaccine composition as claimed in claim 8.

15. A composition as claimed in claim 1, wherein the oil in water emulsion comprises oil droplets which have a diameter which is less than 1 micron.

16. A composition as claimed in claim 1, wherein the oil in water emulsion comprises oil droplets which are in the range of 120 to 750 nm in diameter.

17. A composition as claimed in claim 1, wherein the oil in water

emulsion comprises oil droplets which are in the range of 120 to 600 nm in diameter.

18. A composition as claimed in claim 1, wherein the saponin present in the aqueous phase of the oil in water emulsion is stabilized in its non-hydrolyzed, adjuvant active form.

19. A method as claimed in claim 18, wherein the saponin is QS21.

20. A method as claimed in claims 18 or 19, wherein the sterol is cholesterol.

21. A method as claimed in claim 18, characterised in the oil phase of said oil in water emulsion comprises squalene, said saponin is QS21, and wherein the ratio of squalene:QS21 is substantially 48:1 (w/w).

22. A composition comprising an oil in water emulsion having an oil phase and an aqueous phase, and QS21, the oil phase comprises squalene and cholesterol and said QS21 is in the aqueous phase of said oil in water emulsion, wherein the ratio of QS21:cholesterol is in the range of 1:1 to 1:10 (w/w).

23. A composition as claimed in claim 22, wherein the ratio of squalene:QS21 is in the range from 1:1 to 250:1 (w/w).

24. A composition as claimed in claims 22, wherein the ratio of squalene:QS21 is substantially 48:1 (w/w).

25. A method of treating an individual susceptible to or suffering from a disease by the administration of a composition as claimed in any one of claims 1 to 7.

L3 ANSWER 14 OF 17 USPATFULL on STN
2001:233138 Vaccines.
Friede, Martin, Cardiff, CA, United States
Garcon, Nathalie, Rixensart, Belgium
SmithKline Beecham Biologicals s.a. (U.S. corporation)
US 2001053365 A1 20011220
APPLICATION: US 2001-819464 A1 20010328 (9)
PRIORITY: GB 1995-8326 19950425
GB 1996-910019 19960401
GB 1996-20795 19961005
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. An adjuvant composition comprising an immunostimulatory saponin fraction derived from the bark of Quillaja Saponaria Molina as a single HPLC peak and a sterol, with the proviso that when the adjuvant formulation comprises an ISCOM the saponin is Qs21:

2. An adjuvant composition as claimed in claim 1 wherein the immunologically active saponin fraction is derived from the bark of Quillaja Saponaria Molina is at least 90% pure.

3. An adjuvant composition as claimed in any one of claim 1, wherein the immunologically active saponin fraction derived from the bark of Quillaja Saponaria Molina is QS21.

4. An adjuvant composition as claimed in claim 1 wherein the sterol is in excess weight for weight to the immunologically active saponin fraction.

5. An adjuvant composition as claimed in any one of claim 1 wherein the ratio of saponin:sterol is from 1:100 to 1:1 (w/w).

6. An adjuvant composition as claimed in claim 5 wherein the ratio of saponin:sterol is at least 1:2 (w/w).

7. An adjuvant composition as claimed in claim 6, wherein the ratio of saponin:sterol is 1:5 (w/w).

8. An adjuvant composition as claimed in claim 1, wherein the immunologically active saponin fraction derived from the bark of Quillaja Saponaria Molina is QS17.

9. An adjuvant composition as claimed in claim 1, wherein the sterol is cholesterol.

10. An adjuvant composition as claimed in claim 1, wherein the adjuvant composition is in the form of a vesicle.

11. An adjuvant composition as claimed in claim 10, wherein the adjuvant composition is in the form of a liposome.

12. An adjuvant composition as claimed in claim 11, wherein the adjuvant composition is in the form of a small unilamellar liposome.

13. An adjuvant composition as claimed in claim 10, wherein the adjuvant composition further comprises a phospholipid.

14. An adjuvant composition as claimed in claim 13, wherein the phospholipid is dioleoyl phosphatidylcholine.

15. An adjuvant composition comprising a saponin, a sterol, and a derivative of LPS.

16. An adjuvant composition as claimed in claim 15, wherein the LPS derivative is present in a lipid bilayer membrane.

17. An adjuvant composition as claimed in claim 15, wherein the derivative of LPS is a purified or synthetic lipid A of the following formula: ##STR4## wherein R2 may be H or PO3H2; R3 may be an acyl chain or β-hydroxymyristoyl or a 3-acyloxyacyl residue having the formula: ##STR5##

18. An adjuvant composition as claimed in claim 17, wherein the LPS derivative is 3-O-deacylated monophosphoryl lipid A.

19. An adjuvant composition comprising QS21, 3D-MPL and cholesterol.

20. An adjuvant formulation comprising a purified and stable QS21 saponin which is substantially devoid of hydrolysed QS21

21. An adjuvant formulation comprising 3D-MPL and a liposome, wherein the 3D-MPL is present in the lipid bilayer membrane.

22. An adjuvant composition as claimed in any one of claims 1 to 21, wherein the composition further comprises a carrier.

23. An adjuvant composition as claimed in claim 22, wherein the carrier is an oil in water emulsion or a metallic salt particle.

24. An adjuvant composition comprising a saponin, a sterol and a metallic salt particle.

25. An adjuvant composition as claimed in claim 24, wherein the metallic salt particle is aluminium hydroxide or aluminium phosphate.

26. An adjuvant composition as claimed in claim 24, wherein the saponin is QS21.

27. An immunogenic composition comprising an adjuvant composition as claimed in any one of claims 1 to 21, further comprising an antigen or antigenic composition.

28. An immunogenic composition comprising an adjuvant composition as claimed in claim 22, further comprising an antigen or antigenic composition.

29. A vaccine composition as claimed in any one of claims 1 to 21, further comprising an antigen or antigenic composition.

30. A vaccine composition as claimed in claim 22, further comprising an antigen or antigenic composition.

31. A vaccine as claimed in claim 29, wherein the antigen is derived from any of Human Immunodeficiency Virus, Feline Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium or Toxoplasma.

32. A vaccine as claimed in claim 30, wherein the antigen is derived from any of Human Immunodeficiency Virus, Feline Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C or E, Respiratory

Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium or Toxoplasma.

33. A vaccine as claimed in claim 29 wherein the antigen is a tumour antigen.

34. A vaccine as claimed in claim 30 wherein the antigen is a tumour antigen.

35. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition as claimed in claim 27.

36. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition as claimed in claim 28.

37. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition as claimed in claim 29.

38. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition as claimed in claim 30.

39. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition as claimed in claim 27.

40. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition as claimed in claim 28.

41. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition as claimed in claim 29.

42. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition as claimed in claim 30.

43. A process for making a vaccine composition as claimed in claim 29, comprising admixing an immunologically active saponin fraction and cholesterol with an antigen or antigenic composition.

44. A process for making a vaccine composition as claimed in claim 30, comprising admixing an immunologically active saponin fraction and cholesterol with an antigen or antigenic composition.

45. A method of inducing CTL responses in a mammal comprising administering a vaccine composition as claimed in claim 29.

46. A method of inducing CTL responses in a mammal comprising administering a vaccine composition as claimed in claim 30.

47. A method of reducing the reactogenicity of QS21 containing adjuvant formulations, by the addition of excess sterol to the adjuvant formulation (weight/weight).

48. A method of stabilising QS21 against alkali mediated hydrolysis in QS21 containing adjuvant formulations, by the addition of excess sterol to the adjuvant formulation (weight/weight).

49. A process for the manufacture of an adjuvant formulation comprising making small unilamellar liposomes (SUV) comprising a sterol such as cholesterol, followed by the admixture of a saponin.

L3 ANSWER 15 OF 17 USPATFULL on STN
2000:153268 Vaccines.
Momin, Patricia Marie, Brussels, Belgium
Garcon, Nathalie Marie-Josephe, Wavre, Belgium
SmithKline Beecham Biologicals s.a., Rixensart, Belgium (non-U.S. corporation)
US 6146632 20001114
WO 9517210 19950629
APPLICATION: US 1996-663289 19960702 (8)
WO 1994-EP4246 19941220 19960702 PCT 371 date 19960702 PCT 102(e) date

PRIORITY: GB 1993-26253 19931223
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:

1. A vaccine composition comprising an antigen and/or antigenic composition, QS21, 3-De-O-acylated monophosphoryl lipid A (3D-MPL) and an oil in water emulsion wherein the oil in water emulsion comprises a metabolizable oil, alpha tocopherol and TWEEN 80 (polyoxyethelene sorbitan monooleate).

2. The vaccine composition according to claim 1 wherein said metabolizable oil is squalene.

3. The vaccine composition according to claim 2 wherein the ratio of QS21:3D-MPL is from 1:10 to 10:1.

4. The vaccine composition according to claim 3 wherein said ratio is from 1:1 to 1:2.5.

5. The vaccine composition according to claim 1 wherein said vaccine is capable of invoking a cytolytic T cell response to said antigen and/or antigenic composition in a mammal.

6. The vaccine composition according to claim 1 wherein said vaccine is capable of stimulating interferon γ production.

7. An immunogenic composition comprising an antigen and/or antigenic composition, QS21, 3-De-O-acylated monophosphoryl lipid A (3D-MPL) and an oil in water emulsion wherein the oil in water emulsion comprises a metabolizable oil, alpha tocopherol and TWEEN 80 (polyoxyethelene sorbitan monooleate) wherein said antigen and/or antigenic composition is selected from the group consisting of Herpes Simplex Virus type 1, Herpes Simplex Virus type 2, Human cytomegalovirus, Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis E, Respiratory Syncytial Virus, Human papilloma Virus, Influenza Virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium and Toxoplasma.

8. An immunogenic composition comprising an antigen and/or antigenic composition, QS21, 3-De-O-acylated monophosphoryl lipid A (3D-MPL) and an oil in water emulsion wherein the oil in water emulsion comprises a metabolizable oil, alpha tocopherol and TWEEN 80 (polyoxyethelene sorbitan monooleate) wherein the antigen and/or antigenic composition is a tumor antigen.

9. A method of treating a mammal having a viral, bacterial or parasitic infection by administering a therapeutically safe and effective amount of the immunogenic composition of claim 7.

10. A method of treating a mammal susceptible to a viral, bacterial or parasitic infection by administering a prophylactically safe and effective amount of the immunogenic composition of claim 7.

11. A method of treating a mammal having cancer by administering a therapeutically safe and effective amount of the immunogenic composition of claim 8.

12. A process for making a vaccine according to claim 1 comprising admixing QS21, 3D-MPL and the oil and water emulsion with an antigen or antigen composition.

13. An immunogenic composition comprising an antigen and/or antigenic composition, QS21, 3-De-O-acylated monophosphoryl lipid A (3D-MPL) and an oil in water emulsion wherein the oil in water emulsion comprises a metabolizable oil, alpha tocopherol and TWEEN 80 (polyoxyethelene sorbitan monooleate) wherein said antigen and/or antigenic composition is selected from the group consisting of Human Immunodeficiency Virus and Feline Immunodeficiency Virus.

14. A method of treating a mammal having a viral infection by administering a therapeutically safe and effective amount of the immunogenic composition of claim 13.

15. A method of treating a mammal susceptible to a viral infection by administering a prophylactically safe and effective amount of the immunogenic composition of claim 13.

L3 ANSWER 16 OF 17 USPATFULL on STN
1999:132238 Hepatitis B vaccine.

Hauser, Pierre, Chaumont Gistoux, Belgium
Garcon, Nathalie Marie-Josephe Claude, Wavre, Belgium
Desmons, Pierre, Nivelles, Belgium
SmithKline Beecham Biologicals S.A., Rixensart, Belgium (non-U.S. corporation)
US 5972346 19991026
WO 9626741 19960906
APPLICATION: US 1997-894643 19971027 (8)
WO 1996-EP681 19960215 19971027 PCT 371 date 19971027 PCT 102(e) date
PRIORITY: GB 1995-3863 19950225
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:

1. A vaccine composition comprising a hepatitis B antigen, 3-O-deacylated monophosphoryl lipid A and aluminum phosphate.

2. A vaccine composition as claimed in claim 1 wherein the antigen comprises Hepatitis B surface antigen (HBsAg) or a fragment thereof.

3. A vaccine composition as claimed in claim 2 wherein the antigen is HBsAg and comprises the S antigen of HBsAg.

4. A vaccine composition as claimed in claim 3 wherein the antigen is HBsAg and comprises a pre-S sequence and the S-antigen.

5. A vaccine composition as claimed in claim 3 wherein the HBsAg is a composite particle which contains a modified L protein of hepatitis B virus having an amino acid sequence comprising residues 12-52 followed by residues 133-145 followed by residues 175-400 of the L protein and the S-protein of HBsAg.

6. A vaccine composition as claimed in claim further comprising a component selected from a group consisting of a DTP (diptheria-tetanus-pertussis) HBsAg combination, an Hib-HBsAg combination, a DTP-Hib-HBsAg combination and an IPV (inactivated polio vaccine)-DTP-Hib-HBsAg combination.

7. A vaccine composition as claimed in claim 1 wherein the 3-O-deacylated monophosphoryl lipid A is present in the range 10 µg-100 µg per dose.

8. A method of inducing neutralising antibody titres in the range of 10 mU for hepatitis B in a human susceptible to or suffering from hepatitis B infection by administering the vaccine composition as claimed in claim 1.

9. A method of preventing hepatitis B infections in humans which comprises administering an effective amount of a vaccine composition as claimed in claim 1.

10. A method of treating a human subject suffering from an ongoing hepatitis B infection comprising administering an effective amount of a therapeutic vaccine composition as claimed in claim 1.

11. A process for the production of a vaccine composition as claimed in claim 1 comprising absorbing Hepatitis B surface antigen on to aluminium phosphate and then adding 3-O-deacylated monophosphoryl lipid A.

12. A vaccine composition as claimed in claim 4 wherein the HBsAg is a composite particle which contains a modified L protein of hepatitis B virus having an amino acid sequence comprising residues 12-52 followed by residues 133-145 followed by residues 175-400 of the L protein and the S-protein of HBsAg.

L3 ANSWER 17 OF 17 USPATFULL on STN
95:98944 Liposomes that provide thymic dependent help to weak vaccine antigens.
Six, Howard R., East Stroudburg, PA, United States
Garcon, Nathalie B., Rixensart, Belgium
Research Development Foundation, Carson City, NV, United States (U.S. corporation)
US 5464630 19951107
APPLICATION: US 1995-380213 19950130 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:

1. A liposomal immunogenic carrier for antigens, consisting essentially of a liposome-forming lipid, a 'N-2(2,4 dinitrophenyl E-aminocaproylphosphatidylethanolamine' target antigen and at least one

helper peptide having at least one T-helper cell recognition site, wherein said T-helper cell recognition site is in or on the liposome and wherein said target antigen and helper peptide are not bound to each other, and wherein said helper peptide is HA_2 polypeptide subunit of influenza virus.

2. A liposomal immunogenic carrier according to claim 1 comprising a lipid selected from the group consisting of a phosphatidyl ether, phosphatidyl ester, cerebroside, ganglioside, sphingomyelin, and mixtures thereof.

3. A liposomal immunogenic carrier according to claim 1, wherein said helper peptide is associated inside the liposome via hydrophobic interactions.

4. A liposomal immunogenic carrier according to claim 1, wherein the helper peptide is associated inside the liposome by a covalent link to a liposome-forming lipid.

5. A liposomal immunogenic carrier according to claim 1, wherein the carrier comprises approximately one HA_2 molecule per 120,000 lipid molecules.

6. A method for eliciting an immune response in mammals against a target antigen comprising administering to an animal an immunogenic amount of a liposomal immunogenic carrier of claim 1.

7. A method for eliciting an immune response in mammals against a target antigen comprising administering to an animal an immunogenic amount of a liposomal immunogenic carrier of claim 2.

8. A method for eliciting an immune response mammals against a target antigen comprising administering to an animal an immunogenic amount of a liposomal immunogenic carrier of claim 3.

9. A method for eliciting an immune response in mammals against a target antigen comprising administering to an animal an immunogenic amount of a liposomal immunogenic carrier of claim 4.

10. A method for eliciting an immune response mammals against a target antigen comprising administering to an animal an immunogenic amount of a liposomal immunogenic carrier of claim 5.

11. A liposomal immunogenic carrier according to claim 2, wherein phosphatidyl esters are selected from the group consisting of phosphatidylethanolamine and phosphatidylcholine

12. A liposomal immunogenic carrier according to claim 2, wherein said steroid is cholesterol.

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=> d his

     (FILE 'HOME' ENTERED AT 00:18:42 ON 13 NOV 2006)

     FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006
                E GARCON NATALIE/IN
L1            25 S E4-E7
L2             8 S L1 AND (CPG)
L3            17 S L1 NOT L2

=> e friede martin/in
E1             1     FRIEDE KLAUS/IN
E2             1     FRIEDE MANFRED/IN
E3            17 --> FRIEDE MARTIN/IN
E4             7     FRIEDE MATTHEW D/IN
E5             3     FRIEDE PATRICK W/IN
E6             1     FRIEDE PETER/IN
E7             1     FRIEDE PETRA/IN
E8             1     FRIEDE ROTEM/IN
E9             2     FRIEDE STEVE G/IN
E10            1     FRIEDE WOLFGANG/IN
E11            1     FRIEDEBACH A HANS/IN
E12           11     FRIEDEBACH ADOLF H/IN

=> s e3
L4            17 "FRIEDE MARTIN"/IN
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=> s l4 not l1
L5          12 L4 NOT L1

=> d 15,ti,1-12

L5   ANSWER 1 OF 12  USPATFULL on STN
TI     Vaccines containing a saponin and a sterol

L5   ANSWER 2 OF 12  USPATFULL on STN
TI     Epitopes or mimotopes derived from the C-epsilon-3 or C-epsilon-4
       domains of IgE, antagonists thereof, and their therapeutic uses

L5   ANSWER 3 OF 12  USPATFULL on STN
TI     Intranasal influenza virus vaccine

L5   ANSWER 4 OF 12  USPATFULL on STN
TI     Epitopes or mimotopes derived from the C-epsilon-3 or C-epsilon-4
       domains of IgE, antagonists thereof, and their therapeutics uses

L5   ANSWER 5 OF 12  USPATFULL on STN
TI     Stabilized HBc chimer particles as therapeutic vaccine for chronic
       hepatitis

L5   ANSWER 6 OF 12  USPATFULL on STN
TI     Novel compounds and process

L5   ANSWER 7 OF 12  USPATFULL on STN
TI     Stabilized HBc chimer particles as therapeutic vaccine for chronic
       hepatitis

L5   ANSWER 8 OF 12  USPATFULL on STN
TI     Vaccine

L5   ANSWER 9 OF 12  USPATFULL on STN
TI     Epitopes or mimotopes derived from the C-epsilon-3 or C-epsilon-4
       domains of lgE, antagonists thereof, and their therapeutic uses

L5   ANSWER 10 OF 12  USPATFULL on STN
TI     Vaccine adjuvants

L5   ANSWER 11 OF 12  USPATFULL on STN
TI     Method to enhance an immune response of nucleic acid vaccination

L5   ANSWER 12 OF 12  USPATFULL on STN
TI     Method to enhance an immune response of nucleic acid vaccination


=> d 15,cbib,clm,1-12

L5   ANSWER 1 OF 12  USPATFULL on STN
2005:247127 Vaccines containing a saponin and a sterol.
    Claude Garcon, Nathalie Marie-Josephe, Wavre, BELGIUM
    Friede, Martin, Brussels, BELGIUM
    SmithKline Beecham Biologicals s.a. (non-U.S. corporation)
    US 2005214322 A1 20050929
    APPLICATION: US 2004-967395  A1  20041018 (10)
    PRIORITY: GB 1995-8326  19950425
    GB 1995-13107  19950628
    DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM    What is claimed is:
       1.-12. (canceled)

       13. An adjuvant composition comprising a sterol and a substantially pure
       preparation of QS21, characterized in that the adjuvant composition is
       in the form of an ISCOM.

       14. An adjuvant composition according to claim 1, wherein the QS21 is at
       least 90% pure.

       15. An adjuvant composition according to claim 1, wherein the QS21 is at
       least 95% pure.

       16. An adjuvant composition according to claim 1, wherein the QS21 is at
       least 98% pure.


L5   ANSWER 2 OF 12  USPATFULL on STN
2005:247090 Epitopes or mimotopes derived from the C-epsilon-3 or C-epsilon-4
```

domains of IgE, antagonists thereof, and their therapeutic uses.
Dyson, Michael, Cambidge, UNITED KINGDOM
Friede, Martin, Cardiff, CA, UNITED STATES
Greenwood, Judith, Cambridge, UNITED KINGDOM
Hewitt, Ellen, Royston, UNITED KINGDOM
Lamont, Alan, Croydon, UNITED KINGDOM
Mason, Sean, Cambridge, UNITED KINGDOM
Randall, Roger, Colne, UNITED KINGDOM
Turnell, William Gordon, Cambridge, UNITED KINGDOM
Van Mechelen, Marcelle Paulette, Wagnelee, BELGIUM
y de Bassols, Carlotta Vinals, Brussels, BELGIUM
SmithKline Beecham Biologicals, s.a. and Peptide Therapeutics Limited
(non-U.S. corporation)
US 2005214285 A1 20050929
APPLICATION: US 2004-5794 A1 20041207 (11)
PRIORITY: GB 1999-7151 19990329
GB 1999-10537 19990507
GB 1999-10538 19990507
GB 1999-18594 19990807
GB 1999-18603 19990807
GB 1999-21046 19990907
GB 1999-21047 19990907
GB 1999-25619 19991029
GB 1999-27698 19991123
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1-41. (canceled)

42. A peptide, or mimotope thereof of less than 100 amino acids in length, comprising an isolated surface exposed epitope of a Cε2 domain of IgE, wherein said surface exposed epitope of Cε2 is P1 (SEQ ID NO. 1).

43. A peptide as claimed in claim 42 wherein the surface exposed epitope of Cε2 is P2 (SEQ ID NO. 2), or a mimotope thereof.

44. A peptide as claimed in claim 42 wherein the surface exposed epitope of Cε2 is P3 (SEQ ID NO. 3), or a mimotope thereof.

45. A peptide as claimed in claim 42 wherein the surface exposed epitope of Cε2 is P4 (SEQ ID NO. 4), or a mimotope thereof.

46. A peptide as claimed in claim 42 wherein the surface exposed epitope of Cε2 is P5 (SEQ ID NO. 5), or mimotope thereof.

47. A peptide as claimed in claim 42 wherein the surface exposed epitope of Cε2 is P6 (SEQ ID NO. 6), or a mimotope thereof.

48. A peptide as claimed in claim 42 wherein the surface exposed epitope of Cε2 is P7 (SEQ ID NO. 7), or a mimotope thereof.

49. A mimotope as claimed in claim 42 wherein the mimotope is a peptide.

50. The peptide, or mimotope thereof as claimed in claim 42 wherein the isolated epitope is derived from a loop structure of the Cε2 domain of IgE.

51. The peptide, or mimotope thereof as claimed in claim 51, wherein the loop structure of the CP2 domain of IgE is a A-B or a C-D loop.

52. A peptide as claimed in claim 43 wherein the mimotope of P1 is a peptide of the general formula: h x d h h a n a n x y; wherein: h is a hydrophobic amino acid residue; d is an ionic bond donating amino acid residue; a is an acidic amino acid residue; n is an ionically neutral/non-polar amino acid residue; and x is an amino acid.

53. A peptide as claimed in claim 43, wherein the mimotope of P1 is a peptide of the general formula: Q, X_1, M, D, X_1, X_2, X_3 wherein X_1 is selected from V, I, L, M, F or A; X_2 is selected from D or E; and X_3 is selected from L, I, V, M, A or F.

54. A peptide as claimed in claim 43 wherein the mimotope of P1 is selected from the group consisting of P15q (SEQ ID NO. 11), PT1079 (SEQ ID NO. 13), PT1079GS (SEQ ID NO. 15), PT1078 (SEQ ID NO. 16), and PT15 (SEQ ID NO. 8).

55. A peptide as claimed in claim 44, wherein the mimotope of P2 is P16 (SEQ ID NO. 24).

56. A peptide as claimed in claim 45 wherein the mimotope of P3 is P17 (SEQ ID NO. 26).

57. An immunogenic composition for the treatment of allergy comprising the peptide, or mimotope thereof as claimed in claim 42, additionally comprising a carrier molecule.

58. The immunogenic composition as claimed in claim 58, wherein the carrier molecule is selected from Protein D or Hepatitis B core antigen.

59. An immunogenic composition for the treatment of allergy comprising the peptide, or mimotope thereof as claimed in claim 42, wherein the immunogenic composition is a chemical conjugate of the peptide or mimotope thereof.

60. The immunogenic composition as claimed in claim 58, wherein the peptide, or mimotope thereof is presented within the primary sequence of the carrier.

61. The immunogenic composition as claimed in claim 60, wherein the peptide, or mimotope thereof is presented within the primary sequence of the carrier.

62. A vaccine for the treatment of allergy comprising the immunogenic composition as claimed in claim 58, further comprising an adjuvant.

63. A vaccine for the treatment of allergy comprising the immunogenic composition as claimed in claim 59, further comprising an adjuvant.

64. A vaccine for the treatment of allergy comprising the immunogenic composition as claimed in claim 60, further comprising an adjuvant.

65. A vaccine for the treatment of allergy comprising the immunogenic composition as claimed in claim 61, further comprising an adjuvant.

66. A vaccine for the treatment of allergy comprising the immunogenic composition as claimed in claim 62, further comprising an adjuvant.

67. A ligand which is capable of recognizing a surface exposed epitope of the Cε2 domain of IgE, characterized in that the ligand is not PTmAb0005.

68. A ligand as claimed in claim 68, wherein the ligand is PTmAb0011 deposited under the Budapest Treaty patent deposit at ECACC on Mar. 8th, 1999 under Accession No. 99030805.

69. A pharmaceutical composition comprising a ligand which is capable of recognizing a surface exposed epitope of the Cε2 domain of IgE.

70. A pharmaceutical composition as claimed in claim 70 wherein the ligand is capable of recognizing the C-D Loop of the Cε2 domain of IgE.

71. A pharmaceutical composition as claimed in claim 71, wherein the ligand is a monoclonal antibody selected from PTmAb0005 or PTmAb0011.

72. A peptide which is recognized by PTmAb0005 or PTmAb0011.

73. An immunogen comprising a peptide as claimed in claim 73.

74. A method of manufacturing a vaccine comprising the manufacture of an immunogen as claimed in claim 58, and formulating the immunogen with an adjuvant.

75. A method of manufacturing a vaccine comprising the manufacture of an immunogen as claimed in claim 60, and formulating the immunogen with an adjuvant.

76. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a peptide as claimed in any one of claims 42, to the patient.

77. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a vaccine as claimed in claim 63 to the patient.

78. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a vaccine as claimed in claim

64 to the patient.

79. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a vaccine as claimed in claim 65 to the patient.

80. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a vaccine as claimed in claim 66 to the patient.

81. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a vaccine as claimed in claim 67 to the patient.

82. A method of treating a patient suffering from or susceptible to allergy comprising administration of a pharmaceutical composition as claimed in any one of claims 65, to the patient.

83. An immunogenic composition for the treatment of allergy comprising the peptide, or mimotope thereof as claimed in claim 42, wherein the immunogenic composition is expressed as a fusion protein, and a carrier molecule.

84. The immunogenic composition as claimed in claim 84, wherein the peptide, or mimotope thereof is presented within the primary sequence of the carrier.

85. A vaccine for the treatment of allergy comprising the immunogenic composition as claimed in claim 84, further comprising an adjuvant.

L5 ANSWER 3 OF 12 USPATFULL on STN
2005:233012 Intranasal influenza virus vaccine.
Friede, Martin, Cardiff, CA, UNITED STATES
Henderickx, Veronique, Rixensart, BELGIUM
Hermand, Philippe, Rixensart, BELGIUM
Slaoui, Moncef Mohammed, Rixensart, BELGIUM
Thoelen, Stefan Gabriel Jozef, Rixensart, BELGIUM
SmithKline Beecham Biologicals sa (U.S. corporation)
US 2005201946 A1 20050915
APPLICATION: US 2005-119994 A1 20050502 (11)
PRIORITY: GB 2000-16686 20000706
GB 1999-22700 19990924
GB 1999-22703 19990924
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1-32. (canceled)

33. A process for the preparation of a split influenza vaccine, the method comprising the steps of: (i) harvesting of virus-containing material from a culture; (ii) clarification of the harvested material to remove non-virus material; (iii) concentration of the harvested virus; (iv) filtrating the resuspended sediment to separate the whole virus from non-virus material; (v) concentrating the virus by isopycnic centrifugation in a linear sucrose gradient containing thiomersal (vi) splitting of the whole virus using a suitable splitting agent; and (vii) filtration to remove undesired materials.

34. The process as claimed in claim 33, wherein the split influenza vaccine is chosen from the group of: a monovalent influenza vaccine and a multivalent influenza vaccine comprising at least two strains of influenza.

35. The process as claimed in claim 34, wherein the split influenza vaccine is a trivalent vaccine.

36. The process as claimed in claim 33, wherein the virus is grown on embryonated hen eggs, and the harvested material is allantoic fluid.

37. The process as claimed in claims 33, wherein the virus is grown on a suitable cell substrate.

38. The process as claimed in claim 33, wherein the concentration step (iii) is performed by adsorption using CaHPO4, followed by sedimentation for at least 8 hours, removal of the supernatant and resuspension of the virus-containing sediment in an EDTA-Na2 solution.

39. The process as claimed in claim 38, wherein CaHPO4 is used at a

final concentration of 1.5 g to 3.5 g $CaHPO_4$/liter, and resuspension is made by addition of a 0.26 mol/L EDTA-Na_2 solution.

40. The process as claimed in claim 33, wherein step (v) is performed in a linear sucrose gradient (0 to 55%) (w/v) containing 100 µg/ml thiomersal.

41. The process as claimed in claim 33, wherein the splitting agent is 0.7-1% (w/v) sodium deoxycholate and the splitting is made in the presence of up to 0.1% (w/v) Tween 80.

42. The process as claimed in claim 33, wherein the splitting is performed in a further sucrose density gradient centrifugation step.

43. The process as claimed in claim 33, wherein the filtration step (vii) is an ultrafiltration step which concentrates the split virus material.

44. The process as claimed in claim 33, wherein there is at least one sterile filtration step.

45. The process as claimed in claim 44, wherein said at least one sterile filtration step is performed as the final step (viii).

46. The process as claimed in claim 33, wherein an inactivation step is performed prior to the final filtration step (vii).

47. The split influenza vaccine obtained by the process as claimed in claim 33, wherein the vaccine comprises a solution chosen from the group of: (1) sodium deoxycholate, Tween 80, and thiomersal, and (2) sodium deoxycholate, Triton X-100, and thiomersal.

48. The split influenza vaccine obtained by the process as claimed in claim 47, wherein the sodium deoxycholate is at a maximum concentration of 100 µg/ml, the Tween 80 concentration is about 0.10% (w/v), the Triton X-100 concentration is between 0.05% and 0/02% (w/v), and the thiomersal concentration is less than 10 µg/ml.

49. The split influenza vaccine as claimed in claim 47, wherein the sodium deoxycholate concentration is not greater than 0.05% (w/v), the Tween 80 concentration is from 0.01% to 1% (w/v), the Triton X-100 concentration is 0.001% to 0.1% (w/v), and the thiomersal concentration is below 35 µg/ml of vaccine dose.

L5 ANSWER 4 OF 12 USPATFULL on STN
2005:176912 Epitopes or mimotopes derived from the C-epsilon-3 or C-epsilon-4 domains of IgE, antagonists thereof, and their therapeutics uses.
Friede, Martin, Cardiff, CA, UNITED STATES
Mason, Sean, Cambridge, UNITED KINGDOM
Turnell, William Gordon, Cambridge, UNITED KINGDOM
Van Mechelen, Marcelle Paulette, Wagnelee, BELGIUM
de Bassols, Carlotta Vinals y, Brussels, BELGIUM
SmithKline Beecham Biologicals, s.a. (U.S. corporation)
US 2005152892 A1 20050714
APPLICATION: US 2004-4771 A1 20041203 (11)
PRIORITY: GB 1999-17144 19990721
GB 1999-18598 19990807
GB 1999-18599 19990807
GB 1999-18601 19990807
GB 1999-18604 19990807
GB 1999-18606 19990807
GB 1999-25618 19991029
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1-39. (canceled)

40. A peptide comprising an isolated surface exposed epitope of the region spanning Cε3 and Cε4 domains of IgE, wherein the peptide is P7 (SEQ ID NO:3), or a mimotope thereof.

41. A peptide comprising an isolated surface exposed epitope of the Cε4 domain of IgE, wherein the peptide is P8 (SEQ ID NO:4), or a mimotope thereof.

42. A peptide comprising an isolated surface exposed epitope of the Cε4 domain of IgE, wherein the peptide is P9 (SEQ ID NO:5), or a mimotope thereof.

43. A peptide comprising an isolated surface exposed epitope of the Cε4 domain of IgE, wherein the peptide is 4-90N (SEQ ID NO:84), or a mimotope thereof.

44. A peptide as claimed in claim 41, wherein the mimotope of P8 (SEQ ID NO:4) is a peptide of the general formula: P, X_1, X_2, P, X_3, X_4, X_5, X_6, X_5, X_5 wherein; X_1 is an amino acid selected from E, D, N, or Q; X_2 is an amino acid selected from W, Y, or F; X_3 is an amino acid selected from G or A, X_4 is an amino acid selected from S, T, or M; X_5 is an amino acid selected from R or K; and X_6 is an amino acid selected from D or E.

45. A peptide as claimed in claim 44, wherein the mimotope of P8 (SEQ ID NO:4) is a peptide of the general formula P, X_1, X_2, P, G, X_4, R, D, X_5 wherein, X_1 is an amino acid selected from E, D, N, or Q; X_2 is an amino acid selected from W, Y, or F; X_4 is an amino acid selected from S, T, or M; and X_5 is an amino acid selected from R or K.

46. A mimotope as claimed in claim 40 wherein the mimotope is a peptide.

47. An immunogen for the treatment of allergy comprising a peptide or mimotope as claimed in claim 40, additionally comprising a carrier molecule.

48. An immunogen as claimed in claim 47, wherein the carrier molecule is selected from Protein D or Hepatitis B core antigen.

49. An immunogen as claimed in claim 47, wherein the immunogen is a chemical conjugate of the peptide or mimotope, or wherein the immunogen is expressed as a fusion protein.

50. An immunogen as claimed in claim 48, wherein the immunogen is a chemical conjugate of the peptide or mimotope, or wherein the immunogen is expressed as a fusion protein.

51. An immunogen as claimed in claim 47, wherein the peptide or peptide mimotope is presented within the primary sequence of the carrier.

52. An immunogen as claimed in claim 48, wherein the peptide or peptide mimotope is presented within the primary sequence of the carrier.

53. An immunogen as claimed in claim 49, wherein the peptide or peptide mimotope is presented within the primary sequence of the carrier.

54. An immunogen as claimed in claim 50, wherein the peptide or peptide mimotope is presented within the primary sequence of the carrier.

55. A vaccine for the treatment of allergy comprising a peptide or immunogen as claimed in claim 40, further comprising an adjuvant.

56. A vaccine for the treatment of allergy comprising a peptide or immunogen as claimed in claim 47, further comprising an adjuvant.

57. A vaccine for the treatment of allergy comprising a peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is P5 (SEQ ID NO:1), or mimotope thereof, and an adjuvant.

58. A vaccine for the treatment of allergy comprising a peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is P6 (SEQ ID NO:2), or mimotope thereof, and an adjuvant.

59. A vaccine for the treatment of allergy comprising a peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is P200 (SEQ ID NO:6), or mimotope thereof, and an adjuvant.

60. A vaccine for the treatment of allergy comprising a peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is P210 (SEQ ID NO:7), or mimotope thereof, and an adjuvant.

61. A vaccine for the treatment of allergy comprising a peptide comprising an isolated surface exposed epitope of the Cε3 domain

of IgE, wherein the peptide is 2-90N (SEQ ID NO.82), or mimotope thereof, and an adjuvant.

62. A vaccine for the treatment of allergy comprising a peptide comprising an isolated surface exposed epitope of the Cε4 domain of IgE, wherein the peptide is 3-90N (SEQ ID NO:83), or mimotope thereof, and an adjuvant.

63. A vaccine as claimed in claim 57, wherein the peptide is linked to a carrier molecule to form an immunogen.

64. A vaccine as claimed in claim 63, wherein the immunogen carrier molecule is selected from Protein D or Hepatitis B core antigen.

65. A vaccine as claimed in claim 63, wherein the immunogen is a chemical conjugate of the peptide or mimotope, or wherein the immunogen is expressed as a fusion protein.

66. A vaccine as claimed in claim 63, wherein the peptide or peptide mimotope is presented within the primary sequence of the carrier.

67. A vaccine as claimed in claim 64, wherein the peptide or peptide mimotope is presented within the primary sequence of the carrier.

68. A vaccine as claimed in claim 65, wherein the peptide or peptide mimotope is presented within the primary sequence of the carrier.

69. A ligand which is capable of recognizing the peptides as claimed in claim.

70. A ligand as claimed in claim 69, wherein the ligand is selected from P14/23, P14/31 or P14/33; which are deposited as Budapest Treaty patent deposit at ECACC on 26/1/00 under Accession Nos. 00012610, 00012611, 00012612 respectively.

71. A pharmaceutical composition comprising a ligand as claimed in claim 69.

72. A pharmaceutical composition comprising a ligand as claimed in claim 70.

73. A peptide which is capable of being recognized by P14/23, P14/31, or P14/33; which are deposited as Budapest Treaty patent deposit at ECACC on 26/1/00 under Accession Nos. 00012610, 00012611, 00012612 respectively.

74. A vaccine comprising a peptide as claimed in claim 73.

75. A method of manufacturing a vaccine comprising the manufacture of an immunogen as claimed in claim 47, and formulating the immunogen with an adjuvant.

76. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a vaccine as claimed in claim 55, to the patient.

77. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a vaccine as claimed in claim 56, to the patient.

78. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a vaccine as claimed in claim 63, to the patient.

79. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a vaccine as claimed in claim 64, to the patient.

80. A method of treating a patient suffering from or susceptible to allergy comprising administration of a pharmaceutical composition as claimed in claim 71, to the patient.

81. A method of treating a patient suffering from or susceptible to allergy comprising administration of a pharmaceutical composition as claimed in claim 72, to the patient.

L5 ANSWER 5 OF 12 USPATFULL on STN
2004:202974 Stabilized HBc chimer particles as therapeutic vaccine for chronic

hepatitis.
Page, Mark, Allestree, UNITED KINGDOM
Friede, Martin, Cergue, SWITZERLAND
Schmidt, Annette Elisabeth, Planegg, GERMANY, FEDERAL REPUBLIC OF
Stober, Detlef, Muenchen, GERMANY, FEDERAL REPUBLIC OF
US 2004156863 A1 20040812
APPLICATION: US 2003-677074 A1 20031001 (10)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:

1. A method of treating chronic hepatitis comprising (a) administering to a patient chronically infected with hepatitis B virus a T cell-stimulating amount of a vaccine comprising immunogenic particles dissolved or dispersed in a pharmaceutically acceptable diluent, said immunogenic particles comprising recombinant hepatitis B core (HBc) chimeric protein molecules, said chimeric protein molecules being up to about 550 amino acid residues in length and containing (i) an HBc sequence of at least about 125 of the N-terminal 165 amino acid residues of the HBc molecule that includes the HBc sequence of residue positions 4 through about 75 and about 85 through about 140, and optionally includes (a') a peptide-bonded immunogenic epitope at one or more of the N-terminus, in the HBc immunodominant loop and the C-terminus of the chimer, or (b') an insert in the HBc immunodominant loop, said insert having a length of one to about 40 amino acid residues and containing a chemically-reactive linker residue for a conjugated hapten, (ii) one or both of (a') one to three cysteine residues at an amino acid position of the chimer molecule corresponding to amino acid position -20 to about +1 from the N-terminus of the HBc sequence of SEQ ID NO:1 [N-terminal cysteine residue(s)] in a sequence other than that of the HBc precore sequence and (b') one to about three cysteine residues toward the C-terminus of the molecule from the C-terminal residue of the HBc sequence and within about 30 residues from the C-terminus of the chimer molecule [C-terminal cysteine residue(s)], said chimer molecule (a') containing no more than about 20 percent conservatively substituted amino acid residues in the HBc sequence, (b') self-assembling into particles that upon expression in a host cell are substantially free of binding to nucleic acids, and said particles being more stable than are particles formed from otherwise identical HBc chimer molecules that are free of any above-mentioned C-terminal cysteine residue(s) or N-terminal cysteine residue(s) or in which a C-terminal or an N-terminal cysteine residue(s) present in a contemplated chimer molecule is (are) replaced by another residue; and (b) maintaining said patient for a time sufficient to induce T cells activated against HBc.

2. The method according to claim 1 wherein said peptide-bonded immunogenic epitope or a heterologous linker residue for a conjugated epitope is an immunogenic epitope.

3. The method according to claim 2 wherein said immunogenic epitope is a B cell epitope.

4. The method according to claim 3 wherein said recombinant HBc chimer protein molecule contains a second immunogenic epitope peptide-bonded to the N-terminus, in the HBc immunodominant loop or to the C-terminus of the chimer at a position different from that to which the first-named immunogenic epitope was bonded.

5. The method according to claim 3 wherein said B cell epitope is peptide-bonded at a position in the HBc sequence between amino acid residues 76 and 85, and at least 5 residues of the HBc sequence of positions 76 through 85 are present.

6. The method according to claim 5 wherein the HBc sequence between amino acid residues 76 and 85 is present, but interrupted by said B cell epitope.

7. The method according to claim 2 wherein said recombinant HBc chimer protein molecule further includes a peptide-bonded immunogenic T cell epitope peptide-bonded to the N-terminus, in the HBc immunodominant loop or to the C-terminus of the chimer at a position different from that to which the first-named immunogenic epitope was bonded.

8. The method according to claim 7 wherein said T cell immunogenic epitope is peptide-bonded to the C-terminal HBc amino acid residue.

9. The method according to claim 8 wherein at least one of said C-terminal cysteine residue(s) is present.

10. The method according to claim 1 wherein said chimer contains the

uninterrupted HBc amino acid residue sequence of position 4 through at least position 140, plus a cysteine residue at the N-terminus of the HBc chimer protein molecule.

11. The method according to claim 10 wherein said chimer contains the uninterrupted HBc amino acid residue sequence of position 4 through position 149.

12. The method according to claim 1 wherein said chimer contains a heterologous linker residue for a conjugated epitope present in the HBc immunodominant loop.

13. The method according to claim 12 wherein said heterologous linker residue for a conjugated epitope is peptide-bonded at a position in the HBc sequence between amino acid residues 76 and 85, and at least 4 residues of the HBc sequence of positions 76 through 85 are present.

14. The method according to claim 13 wherein the HBc sequence between amino acid residues 76 and 85 is present, but interrupted by said heterologous linker residue for a conjugated epitope.

15. The method according to claim 14 wherein said recombinant HBc chimer protein molecule contains the HBc amino acid residue sequence of position 4 through at least position 140.

16. The method according to claim 15 wherein said chimer contains the HBc amino acid residue sequence of position 4 through position 149.

17. The method according to claim 16 wherein said heterologous linker residue for a conjugated epitope is selected from the group consisting of a lysine, aspartic acid, glutamic acid, cysteine and a tyrosine residue.

18. A method of treating chronic hepatitis comprising administering to a patient having a chronic hepatitis B virus infection a T cell-stimulating amount of vaccine comprised of an immunogenic effective amount of immunogenic particles dissolved or dispersed in a pharmaceutically acceptable diluent, said immunogenic particles being comprised of recombinant hepatitis B virus core (HBc) protein chimer molecules that have a length of about 135 to about 525 amino acid residues and contain four peptide-linked amino acid residue sequence domains from the N-terminus that are denominated Domains I, II, III and IV, wherein (i) Domain I comprises about 71 to about 110 amino acid residues whose sequence includes (a') at least the sequence of the residues of position 5 through position 75 of HBc, (b') zero to three cysteine residues at an amino acid position of the chimer molecule corresponding to amino acid position -20 to about +1 from the N-terminus of the HBc sequence of SEQ ID NO:1 [N-terminal cysteine residue(s)] in a sequence other than that of the HBc precore sequence, and (c') an optional immunogenic epitope containing up to about 30 amino acid residues peptide-bonded to one of HBc residues 2-4; (ii) Domain II comprises about 5 to about 250 amino acid residues peptide-bonded to HBc residue 75 of Domain I in which (a') zero to all residues in the sequence of HBc positions 76 through 85 are present peptide-bonded to (b') an optionally present sequence of one to about 245 amino acid residues that constitute an immunogenic epitope or a heterologous linker residue for a conjugated epitope; (ii) Domain III is an HBc sequence from position 86 through position 135 peptide-bonded to residue 85 of Domain II; and (iv) Domain IV comprises (a') five through fourteen residues of an HBc amino acid residue sequence from position 136 through 149 peptide-bonded to the residue of position 135 of Domain III, (b') zero to three cysteine residues [C-terminal cysteine residue(s)] within about 30 residues from the C-terminus of the chimer molecule, and (c') zero to about 100 amino acid residues in an immunogenic sequence heterologous to HBc from position 165 to the C-terminus, said chimer molecule (i) having an amino acid residue sequence in which no more than about 10 percent of the amino acid residues are substituted in the HBc sequence of the chimer, (ii) self-assembling into particles on expression in a host cell and (iii) containing at least one N-terminal cysteine residue or C-terminal cysteine residue, said particles being substantially free of binding to nucleic acids and being more stable than are particles formed from otherwise identical HBc chimer molecules that are (i) free of any above-mentioned C-terminal cysteine residue(s) or N-terminal cysteine residue(s) or (ii) in which said cysteine residue(s) of (iii) present in a contemplated chimer molecule is (are) replaced by another residue.

19. The method according to claim 18 wherein said recombinant HBC chimer protein molecule contains two immunogenic epitopes.

20. The method according to claim 19 wherein said recombinant HBc chimer protein molecule contains two immunogenic epitopes that are present in Domains I and II, II and IV or I and IV.

21. The method according to claim 19 wherein one of said two immunogenic epitopes is a B cell epitope.

22. The method according to claim 19 wherein one of said two immunogenic epitopes is a T cell epitope.

23. The method according to claim 19 wherein one of said two immunogenic epitopes are the same or different T cell epitopes.

24. The method according to claim 18 wherein said Domain I includes immunogenic epitope peptide-bonded to one of HBc residues 2-4 and said epitope is a T cell epitope.

25. The method according to claim 18 wherein Domain II contains a immunogenic epitope and said epitope is a B cell epitope.

26. The method according to claim 18 wherein said sequence heterologous to HBc from position 165 to the C-terminus is an immunogenic T cell epitope peptide-bonded to one of HBC residues 140-149.

27. The method according to claim 18 wherein Domain II contains a heterologous linker residue for a conjugated epitope.

28. The method according to claim 24 wherein said recombinant HBc chimer protein molecule contains one to three C-terminal cysteine residue(s) within about 30 residues of the C-terminus of the chimer molecule.

29. The method according to claim 28 wherein said recombinant HBc chimer protein molecule contains an immunogenic epitope present in Domain II that is a B cell epitope.

30. The method according to claim 29 wherein said B cell epitope contains 6 to about 50 amino acid residues.

31. The method according to claim 29 wherein said B cell epitope contains 20 to about 30 amino acid residues.

32. The method according to claim 28 wherein said recombinant HBc chimer protein molecule contains 1 cysteine residue within about 30 residues from the C-terminus of the chimer molecule.

33. The method according to claim 29 wherein the HBc sequence between amino acid residues 76 and 85 is present, but interrupted by said immunogenic epitope.

34. The method according to claim 32 wherein said cysteine residue is located within about five amino acid residues of the C-terminus of the chimer protein molecule.

35. The method according to claim 18 wherein said sequence heterologous to HBc from position 165 to the C-terminus is an immunogenic T cell epitope peptide-bonded to one of HBc residues 140-149.

36. The method according to claim 18 wherein said immunogenic epitope or heterologous linker residue for a conjugated epitope is a heterologous linker residue for a conjugated epitope.

37. The method according to claim 36 wherein said heterologous linker residue for a conjugated epitope is selected from the group consisting of a lysine, aspartic acid, glutamic acid, cysteine and a tyrosine residue.

38. The method according to claim 37 wherein said recombinant HBc chimer protein molecule contains a single cysteine residue at the C-terminus of the HBc chimer protein molecule.

39. A method of treating chronic hepatitis comprising administering to a patient having a chronic hepatitis B virus infection a T cell-stimulating amount of a vaccine comprised of an immunogenic effective amount of immunogenic particles dissolved or dispersed in a pharmaceutically acceptable diluent, said immunogenic particles being comprised of recombinant hepatitis B virus core (HBc) protein chimer molecules with a length of about 170 to about 250 amino acid residues that contains four peptide-linked amino acid residue sequence domains

from the N-terminus that are denominated Domains I, II, III and IV, wherein (a) Domain I comprises about the sequence of the residues of position 4 through position 75 of HBc as well as a first sequence of up to about 25 residues in a first sequence peptide-bonded to the amino-terminal HBC residue of said sequence, said sequence of up to about 25 residues containing zero or one cysteine residue at an amino acid position of the chimer molecule corresponding to amino acid position -14 to about +1 from the N-terminus of the HBc sequence of SEQ ID NO:1 [N-terminal cysteine residue]; (b) Domain II comprises about 5 to about 55 amino acid residues peptide-bonded to HBc residue 75 of Domain I in which at least 4 residues in a sequence of HBc positions 76 through 85 are present peptide-bonded to an optional second sequence heterologous to HBc at positions 76 through 85 of up to about 50 amino acid residues; (c) Domain III is an HBc sequence from position 86 through position 135 peptide-bonded to residue 85 of Domain II; and d) Domain IV comprises (i) 5 through fourteen residues of a HBc amino acid residue sequence from position 136 through 149 peptide-bonded to the residue of position 135 of Domain III, (ii) zero or one cysteine residue [C-terminal cysteine residue] within about 30 residues of the C-terminus of the chimer molecule, and (iii) zero to about 50 amino acid residues in a third sequence heterologous to HBc from position 165 to the C-terminus, said chimer molecules (i) self-assembling into particles on expression in a host cell, (ii) including at least one or the other of said N-terminal cysteine residue or C-terminal cysteine residue and (iii) having an amino acid residue sequence in which no more than about 5 percent of the amino acid residues are substituted in the HBc sequence of the chimer relative to the sequence shown in the HBc sequence of SEQ ID NO:1, said particles exhibiting a ratio of absorbance at 280 nm to 260 nm of about 1.2 to about 1.7 and being more stable than are particles formed from otherwise identical HBc chimer molecules that lack said N-terminal cysteine residue or C-terminal cysteine residue that is present or in which the N-terminal cysteine or C-terminal cysteine residue present in the chimer molecule is replaced by another residue.

40. The method according to claim 39 wherein said second sequence of Domain II defines a B cell epitope.

41. The method according to claim 40 wherein said second sequence contains 15 to about 50 amino acid residues.

42. The method according to claim 40 wherein said second sequence contains 20 to about 30 amino acid residues.

43. The method according to claim 40 wherein the HBc sequence between amino acid residues 76 and 85 is present, but interrupted by said second sequence.

44. The method according to claim 40 wherein said B cell epitope is an amino acid sequence present in a pathogen selected from the group consisting of Streptococcus pneumonia, Cryptosporidium parvum, HIV, foot-and-mouth disease virus, influenza virus, Yersinia pestis, Haemophilus influenzae, Moraxella catarrhalis, Porphyromonas gingivalis, Trypanosoma cruzi, Plasmodium falciparum, Plasmodium vivax, Plasmodium berghi, Plasmodium yoelli, Streptococcus sobrinus, Shigella flexneri, RSV, Plasmodium Entamoeba histolytica, Schistosoma japonicum, Schistosoma mansoni, HBV and ebola virus.

45. The recombinant HBc chimer protein molecule according to claim 40 wherein said sequence heterologous to HBc from position 165 to the C-terminus is an immunogenic T cell epitope peptide-bonded to one of HBc residues 140-149.

46. The recombinant HBc chimer protein molecule according to claim 45 wherein said T cell epitope is from HBV.

47. The recombinant HBc chimer protein molecule according to claim 40 wherein said N-terminal cysteine residue is located within about five amino acid residues of the N-terminal of the chimer protein molecule.

48. A method of enhancing the production of one or more of gamma-producing CD 8+, CD 4+ T cells and cytotoxic T lymphocytes against hepatitis B virus that comprises; (a) administering to a patient chronically infected with hepatitis B virus a T cell-stimulating amount of a vaccine comprising immunogenic particles dissolved or dispersed in a pharmaceutically acceptable diluent that contains one or both of (a) an agonist for toll-like receptor-4 (TLR-4), and (b) an agonist for toll-like receptor-9 (TLR-9), said immunogenic particles comprising recombinant hepatitis B core (HBc) chimeric protein molecules, said chimeric protein molecules being up to about 550 amino acid residues in

length and containing (i) an HBc sequence of at least about 125 of the N-terminal 165 amino acid residues of the HBc molecule that includes the HBc sequence of residue positions 4 through about 75 and about 85 through about 140, and optionally includes (a') a peptide-bonded immunogenic epitope at one or more of the N-terminus, in the HBc immunodominant loop and the C-terminus of the chimer, or (b') an insert in the HBc immunodominant loop, said insert having a length of one to about 40 amino acid residues and containing a chemically-reactive linker residue for a conjugated hapten, (ii) one or both of (a') one to three cysteine residues at an amino acid position of the chimer molecule corresponding to amino acid position -20 to about +1 from the N-terminus of the HBc sequence of SEQ ID NO:1 [N-terminal cysteine residue(s)] in a sequence other than that of the HBc precore sequence and (b') one to about three cysteine residues toward the C-terminus of the molecule from the C-terminal residue of the HBc sequence and within about 30 residues from the C-terminus of the chimer molecule [C-terminal cysteine residue(s)], said chimer molecule (a') containing no more than about 20 percent conservatively substituted amino acid residues in the HBc sequence, (b') self-assembling into particles that upon expression in a host cell are substantially free of binding to nucleic acids, and said particles being more stable than are particles formed from otherwise identical HBc chimer molecules that are free of any above-mentioned C-terminal cysteine residue(s) or N-terminal cysteine residue(s) or in which a C-terminal or an N-terminal cysteine residue(s) present in a contemplated chimer molecule is (are) replaced by another residue; and (b) maintaining said patient for a time sufficient to induce T cells activated against HBc.

49. The method according to claim 48 wherein said agonist for TLR-4 is structurally related to monophosphoryl lipid A.

50. The method according to claim 49 wherein said agonist structurally related to monophosphoryl lipid A is an aminoalkyl glucosamide phosphate.

51. The method according to claim 48 wherein said one or both of said TLR-4 agonist and said TLR-9 agonist are admixed with said pharmaceutically acceptable diluent and said immunogenic particles.

L5 ANSWER 6 OF 12 USPATFULL on STN
2004:39564 Novel compounds and process.
Friede, Martin, Rixensart, BELGIUM
Mason, Sean, Cambridge, UNITED KINGDOM
Turnell, William Gordon, Cambridge, UNITED KINGDOM
Y De Bassols, Carlota Vinals, Rixensart, BELGIUM
US 20040030106 A1 20040212
APPLICATION: US 2003-362527 A1 20030730 (10)
WO 2001-EP9576 20010817
PRIORITY: GB 2000-207717 20000822
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. A process for the manufacture of a vaccine immunogen comprising conjugating a disulphide bridge cyclised peptide to an immunogenic carrier comprising, (a) adding to a disulphide cyclised peptide a moiety comprising a reactive group which is capable of forming thio-ether linkages with thiol bearing carriers, and (b) reacting the activated cyclised peptide thus formed with a thiol bearing immunogenic carrier.

2. A process as claimed in claim 1 wherein the reactive group capable of forming thio-ether linkages with thiol bearing carriers is a maleimide group.

3. A process as claimed in claim 1 wherein the disulphide bridge cyclised peptide is derived from human IgE.

4. A process as claimed in claim 3, wherein the human IgE peptide is selected from any one of SEQ ID NOs. 1 to 328.

5. A process as claimed in claim 1, wherein the carrier is selected from Haemophilus Influenzae Protein D, BSA, Keyhole limpet Haemocyanin (KLH), serum albumins such as bovine serum albumin (BSA), inactivated bacterial toxins such as tetanus or diptheria toxins (TT and DT), or recombinant fragments thereof (for example, Domain 1 of Fragment C of TT, or the translocation domain of DT), or the purified protein derivative of tuberculin (PPD).

6. A disulphide bridge cyclised IgE peptide maleimide derivative.

7. Use of a peptide derivative as claimed in claim 6, in the manufacture of a medicament for the treatment of allergy.

8. A conjugate suitable for use in a vaccine, of formula (I): ##STR3## wherein, carrier is an immunogenic carrier molecule, X is either a linker or a bond, Y is either a linker or a bond, and P is a disulphide bridge cyclised peptide.

9. A conjugate as claimed in claim 8 wherein P is selected from the following group SEQ ID NO.s 99, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, and 328.

10. A vaccine composition comprising the product of the process claimed in any one of claims 1 to 5, and a suitable adjuvant or carrier.

11. A vaccine composition comprising a conjugate as claimed in claim 8 or 9, and a suitable adjuvant or carrier.

12. A vaccine as claimed in claim 10 or 11, wherein the vaccine is an allergy vaccine.

13. A conjugate as claimed in claim 8 for the treatment of allergy.

L5 ANSWER 7 OF 12 USPATFULL on STN
2003:282304 Stabilized HBc chimer particles as therapeutic vaccine for chronic hepatitis.
Page, Mark, Allestree, UNITED KINGDOM
Friede, Martin, Cardiff, CA, UNITED STATES
US 2003198645 A1 20031023
APPLICATION: US 2003-372076 A1 20030221 (10)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. A method of treating chronic hepatitis comprising (a) administering to a patient in need thereof a T cell-stimulating amount of a vaccine comprising immunogenic particles dissolved or dispersed in a pharmaceutically acceptable diluent, said immunogenic particles comprising recombinant hepatitis B core (HBc) chimeric protein molecules, said chimeric protein molecules being up to about 550 amino acid residues in length and containing (i) an HBc sequence of at least about 125 of the N-terminal 165 amino acid residues of the HBc molecule that includes the HBc sequence of residue positions 4 through about 75 and about 85 through about 140, and optionally includes (a') a peptide-bonded immunogenic epitope at one or more of the N-terminus, in the HBc immunodominant loop and the C-terminus of the chimer, or (b') an insert in the HBc immunodominant loop, said insert having a length of one to about 40 amino acid residues and containing a chemically-reactive linker residue for a conjugated hapten, (ii) one or both of (a') one to three cysteine residues at an amino acid position of the chimer molecule corresponding to amino acid position -20 to about +1 from the N-terminus of the HBc sequence of SEQ ID NO:1 [N-terminal cysteine residue(s)] in a sequence other than that of the HBc precore sequence and (b') one to about three cysteine residues toward the C-terminus of the molecule from the C-terminal residue of the HBc sequence and within about 30 residues from the C-terminus of the chimer molecule [C-terminal cysteine residue(s)], said chimer molecule (a') containing no more than about 20 percent conservatively substituted amino acid residues in the HBc sequence, (b') self-assembling into particles that upon expression in a host cell are substantially free of binding to nucleic acids, and said particles being more stable than are particles formed from otherwise identical HBc chimer molecules that are free of any above-mentioned C-terminal cysteine residue(s) or N-terminal cysteine residue(s) or in which a C-terminal or an N-terminal cysteine residue(s) present in a contemplated chimer molecule is(are) replaced by another residue; and (b) maintaining said patient for a time sufficient to induce T cells activated against HBc.

2. The method according to claim 1 wherein said peptide-bonded immunogenic epitope or a heterologous linker residue for a conjugated epitope is an immunogenic epitope.

3. The method according to claim 2 wherein said immunogenic epitope is a B cell epitope.

4. The method according to claim 3 wherein said recombinant HBc chimer protein molecule contains a second immunogenic epitope peptide-bonded to

the N-terminus, in the HBc immunodominant loop or to the C-terminus of the chimer at a position different from that to which the first-named immunogenic epitope was bonded.

5. The method according to claim 3 wherein said B cell epitope is peptide-bonded at a position in the HBc sequence between amino acid residues 76 and 85, and at least 5 residues of the HBc sequence of positions 76 through 85 are present.

6. The method according to claim 5 wherein the HBc sequence between amino acid residues 76 and 85 is present, but interrupted by said B cell epitope.

7. The method according to claim 2 wherein said recombinant HBc chimer protein molecule further includes a peptide-bonded immunogenic T cell epitope peptide-bonded to the N-terminus, in the HBc immunodominant loop or to the C-terminus of the chimer at a position different from that to which the first-named immunogenic epitope was bonded.

8. The method according to claim 7 wherein said T cell immunogenic epitope is peptide-bonded to the C-terminal HBc amino acid residue.

9. The method according to claim 8 wherein at least one of said C-terminal cysteine residue(s) is present.

10. The method according to claim 1 wherein said chimer contains the uninterrupted HBc amino acid residue sequence of position 4 through at least position 140, plus a cysteine residue at the N-terminus of the HBc chimer protein molecule.

11. The method according to claim 10 wherein said chimer contains the uninterrupted HBc amino acid residue sequence of position 4 through position 149.

12. The method according to claim 1 wherein said chimer contains a heterologous linker residue for a conjugated epitope present in the HBc immunodominant loop.

13. The method according to claim 12 wherein said heterologous linker residue for a conjugated epitope is peptide-bonded at a position in the HBc sequence between amino acid residues 76 and 85, and at least 4 residues of the HBc sequence of positions 76 through 85 are present.

14. The method according to claim 13 wherein the HBc sequence between amino acid residues 76 and 85 is present, but interrupted by said heterologous linker residue for a conjugated epitope.

15. The method according to claim 14 wherein said recombinant HBc chimer protein molecule contains the HBc amino acid residue sequence of position 4 through at least position 140.

16. The method according to claim 15 wherein said chimer contains the HBc amino acid residue sequence of position 4 through position 149.

17. The method according to claim 16 wherein said heterologous linker residue for a conjugated epitope is selected from the group consisting of a lysine, aspartic acid, glutamic acid, cysteine and a tyrosine residue.

18. A method of treating chronic hepatitis comprising administering to a patient in need thereof a T cell-stimulating amount of vaccine comprised of an immunogenic effective amount of immunogenic particles dissolved or dispersed in a pharmaceutically acceptable diluent, said immunogenic particles being comprised of recombinant hepatitis B virus core (HBc) protein chimer molecules that have a length of about 135 to about 525 amino acid residues and contain four peptide-linked amino acid residue sequence domains from the N-terminus that are denominated Domains I, II, III and IV, wherein (i) Domain I comprises about 71 to about 110 amino acid residues whose sequence includes (a') at least the sequence of the residues of position 5 through position 75 of HBc, (b') zero to three cysteine residues at an amino acid position of the chimer molecule corresponding to amino acid position -20 to about +1 from the N-terminus of the HBc sequence of SEQ ID NO:1 [N-terminal cysteine residue(s)] in a sequence other than that of the HBc precore sequence, and (c') an optional immunogenic epitope containing up to about 30 amino acid residues peptide-bonded to one of HBc residues 2-4; (ii) Domain II comprises about 5 to about 250 amino acid residues peptide-bonded to HBc residue 75 of Domain I in which (a') zero to all residues in the sequence of HBc positions 76 through 85 are present peptide-bonded to

(b') an optionally present sequence of one to about 245 amino acid residues that constitute an immunogenic epitope or a heterologous linker residue for a conjugated epitope; (ii) Domain III is an HBc sequence from position 86 through position 135 peptide-bonded to residue 85 of Domain II; and (iv) Domain IV comprises (a') five through fourteen residues of an HBc amino acid residue sequence from position 136 through 149 peptide-bonded to the residue of position 135 of Domain III, (b') zero to three cysteine residues [C-terminal cysteine residue(s)] within about 30 residues from the C-terminus of the chimer molecule, and (c') zero to about 100 amino acid residues in an immunogenic sequence heterologous to HBc from position 165 to the C-terminus, said chimer molecule (i) having an amino acid residue sequence in which no more than about 10 percent of the amino acid residues are substituted in the HBc sequence of the chimer, (ii) self-assembling into particles on expression in a host cell and (iii) containing at least one N-terminal cysteine residue or C-terminal cysteine residue, said particles being substantially free of binding to nucleic acids and being more stable than are particles formed from otherwise identical HBc chimer molecules that are (i) free of any above-mentioned C-terminal cysteine residue(s) or N-terminal cysteine residue(s) or (ii) in which said cysteine residue(s) of (iii) present in a contemplated chimer molecule is(are) replaced by another residue.

19. The method according to claim 18 wherein said recombinant HBc chimer protein molecule contains two immunogenic epitopes.

20. The method according to claim 19 wherein said recombinant HBc chimer protein molecule contains two immunogenic epitopes that are present in Domains I and II, II and IV or I and IV.

21. The method according to claim 19 wherein one of said two immunogenic epitopes is a B cell epitope.

22. The method according to claim 19 wherein one of said two immunogenic epitopes is a T cell epitope.

23. The method according to claim 19 wherein one of said two immunogenic epitopes are the same or different T cell epitopes.

24. The method according to claim 18 wherein said Domain I includes immunogenic epitope peptide-bonded to one of HBc residues 2-4 and said epitope is a T cell epitope.

25. The method according to claim 18 wherein Domain II contains a immunogenic epitope and said epitope is a B cell epitope.

26. The method according to claim 18 wherein said sequence heterologous to HBc from position 165 to the C-terminus is an immunogenic T cell epitope peptide-bonded to one of HBc residues 140-149.

27. The method according to claim 18 wherein Domain II contains a heterologous linker residue for a conjugated epitope.

28. The method according to claim 24 wherein said recombinant HBc chimer protein molecule contains one to three C-terminal cysteine residue(s) within about 30 residues of the C-terminus of the chimer molecule.

29. The method according to claim 28 wherein said recombinant HBc chimer protein molecule contains an immunogenic epitope present in Domain II that is a B cell epitope.

30. The method according to claim 29 wherein said B cell epitope contains 6 to about 50 amino acid residues.

31. The method according to claim 29 wherein said B cell epitope contains 20 to about 30 amino acid residues.

32. The method according to claim 28 wherein said recombinant HBc chimer protein molecule contains 1 cysteine residue within about 30 residues from the C-terminus of the chimer molecule.

33. The method according to claim 28 wherein the HBc sequence between amino acid residues 76 and 85 is present, but interrupted by said immunogenic epitope.

34. The method according to claim 32 wherein said cysteine residue is located within about five amino acid residues of the C-terminus of the chimer protein molecule.

35. The method according to claim 18 wherein said sequence heterologous to HBc from position 165 to the C-terminus is an immunogenic T cell epitope peptide-bonded to one of HBc residues 140-149.

36. The method according to claim 18 wherein said immunogenic epitope or heterologous linker residue for a conjugated epitope is a heterologous linker residue for a conjugated epitope.

37. The method according to claim 36 wherein said heterologous linker residue for a conjugated epitope is selected from the group consisting of a lysine, aspartic acid, glutamic acid, cysteine and a tyrosine residue.

38. The method according to claim 37 wherein said recombinant HBc chimer protein molecule contains a single cysteine residue at the C-terminus of the HBc chimer protein molecule.

39. A method of treating chronic hepatitis comprising administering to a patient in need thereof a T cell-stimulating amount of a vaccine comprised of an immunogenic effective amount of immunogenic particles dissolved or dispersed in a pharmaceutically acceptable diluent, said immunogenic particles being comprised of recombinant hepatitis B virus core (HBc) protein chimer molecules with a length of about 170 to about 250 amino acid residues that contains four peptide-linked amino acid residue sequence domains from the N-terminus that are denominated Domains I, II, III and IV, wherein (a) Domain I comprises about the sequence of the residues of position 4 through position 75 of HBc as well as a first sequence of up to about 25 residues in a first sequence peptide-bonded to the amino-terminal HBC residue of said sequence, said sequence of up to about 25 residues containing zero or one cysteine residue at an amino acid position of the chimer molecule corresponding to amino acid position -14 to about +1 from the N-terminus of the HBc sequence of SEQ ID NO:1 [N-terminal cysteine residue]; (b) Domain II comprises about 5 to about 55 amino acid residues peptide-bonded to HBc residue 75 of Domain I in which at least 4 residues in a sequence of HBc positions 76 through 85 are present peptide-bonded to an optional second sequence heterologous to HBc at positions 76 through 85 of up to about 50 amino acid residues; (c) Domain III is an HBc sequence from position 86 through position 135 peptide-bonded to residue 85 of Domain II; and d) Domain IV comprises (i) 5 through fourteen residues of a HBc amino acid residue sequence from position 136 through 149 peptide-bonded to the residue of position 135 of Domain III, (ii) zero or one cysteine residue [C-terminal cysteine residue] within about 30 residues of the C-terminus of the chimer molecule, and (iii) zero to about 50 amino acid residues in a third sequence heterologous to HBc from position 165 to the C-terminus, said chimer molecules (i) self-assembling into particles on expression in a host cell, (ii) including at least one or the other of said N-terminal cysteine residue or C-terminal cysteine residue and (iii) having an amino acid residue sequence in which no more than about 5 percent of the amino acid residues are substituted in the HBc sequence of the chimer relative to the sequence shown in the HBc sequence of SEQ ID NO:1, said particles exhibiting a ratio of absorbance at 280 nm to 260 nm of about 1.2 to about 1.7 and being more stable than are particles formed from otherwise identical HBc chimer molecules that lack said N-terminal cysteine residue or C-terminal cysteine residue that is present or in which the N-terminal cysteine or C-terminal cysteine residue present in the chimer molecule is replaced by another residue.

40. The method according to claim 39 wherein said second sequence of Domain II defines a B cell epitope.

41. The method according to claim 40 wherein said second sequence contains 15 to about 50 amino acid residues.

42. The method according to claim 40 wherein said second sequence contains 20 to about 30 amino acid residues.

43. The method according to claim 40 wherein the HBc sequence between amino acid residues 76 and 85 is present, but interrupted by said second sequence.

44. The method according to claim 40 wherein said B cell epitope is an amino acid sequence present in a pathogen selected from the group consisting of Streptococcus pneumonia, Cryptosporidium parvum, HIV, foot-and-mouth disease virus, influenza virus, Yersinia pestis, Haemophilus influenzae, Moraxella catarrhalis, Porphyromonas gingivalis, Trypanosoma cruzi, Plasmodium falciparum, Plasmodium vivax, Plasmodium berghi, Plasmodium yoelli, Streptococcus sobrinus, Shigella flexneri,

RSV, Plasmodium Entamoeba histolytica, Schistosoma japonicum, Schistosoma mansoni, HBV and ebola virus.

45. The recombinant HBc chimer protein molecule according to claim 40 wherein said sequence heterologous to HBc from position 165 to the C-terminus is an immunogenic T cell epitope peptide-bonded to one of HBc residues 140-149.

46. The recombinant HBc chimer protein molecule according to claim 45 wherein said T cell epitope is from HBV.

47. The recombinant HBc chimer protein molecule according to claim 40 wherein said N-terminal cysteine residue is located within about five amino acid residues of the N-terminal of the chimer protein molecule.

L5 ANSWER 8 OF 12 USPATFULL on STN
2003:243820 Vaccine.
Friede, Martin, Farnham, UNITED KINGDOM
Mason, Sean, Cambridge, UNITED KINGDOM
Turnell, William G., Cambridge, UNITED KINGDOM
Vinals y de Bassols, Carlota, Rixensart, BELGIUM
Van Mechelen, Marcelle Paulette, Rixensart, BELGIUM
SmithKline Beecham Biologicals s.a. (non-U.S. corporation)
US 2003170229 A1 20030911
APPLICATION: US 2002-304443 A1 20021126 (10)
PRIORITY: GB 1999-4408 19990225
GB 1999-17144 19990721
GB 1999-18598 19990807
GB 1999-18599 19990807
GB 1999-18601 19990807
GB 1999-18604 19990807
GB 1999-18606 19990807
GB 1999-25618 19991029
GB 1999-4405 19990225
GB 1999-7151 19990329
GB 1999-10537 19990507
GB 1999-10538 19990507
GB 1999-18594 19990807
GB 1999-18603 19990807
GB 1999-21046 19990907
GB 1999-21047 19990907
GB 1999-25619 19991029
GB 1999-27698 19991123
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:

1. A peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is P5 (SEQ ID No. 1), or mimotope thereof.

2. A peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is P6 (SEQ ID No. 2), or mimotope thereof.

3. A peptide comprising an isolated surface exposed epitope of the region spanning Cε3 and Cε4 domains of IgE, wherein the peptide is P7 (SEQ ID No. 3), or mimotope thereof.

4. A peptide comprising an isolated surface exposed epitope of the Cε4 domain of IgE, wherein the peptide is P8 (SEQ ID No. 4), or mimotope thereof.

5. A peptide comprising an isolated surface exposed epitope of the Cε4 domain of IgE, wherein the peptide is P9 (SEQ ID No. 5), or mimotope thereof.

6. A peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is P200 (SEQ ID No. 6), or mimotope thereof.

7. A peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is P210 (SEQ ID No. 7), or mimotope thereof.

8. A peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is 2-90N (SEQ ID No. 82), or mimotope thereof.

9. A peptide comprising an isolated surface exposed epitope of the Cε4 domain of IgE, wherein the peptide is 3-90N (SEQ ID No. 83), or mimotope thereof.

10. A peptide comprising an isolated surface exposed epitope of the Cε4 domain of IgE, wherein the peptide is 4-90N (SEQ ID No. 84), or mimotope thereof.

11. A mimotope as claimed in any one of claims 1 to 10 wherein the mimotope is a peptide.

12. A peptide as claimed in claim 4, wherein the mimotope of P8 is a peptide of the general formula: P, X_1, X_2, P, X_3, X_4, X_5, X_6, X_5, X_5 wherein; X_1 is an amino acid selected from E, D, N, or Q; X_2 is an amino acid selected from W, Y, or F; X_3 is an amino acid selected from G or A, X_4 is an amino acid selected from S, T or M; X_5 is an amino acid selected from R or K; and X_6 is an amino acid selected from D or E.

13. A peptide as claimed in claim 12, wherein the mimotope of P8 is a peptide of the general formula P, X_1, X_2, P, G, X_4, R, D, X_5, X_5 wherein; X_1 is an amino acid selected from E, D, N, or Q; X_2 is an amino acid selected from W, Y, or F; X_4 is an amino acid selected from S, T or M; X_5 is an amino acid selected from R or K; and X_6 is an amino acid selected from D or E.

14. An immunogen for the treatment of allergy comprising a peptide or mimotope as claimed in any one of claims 1 to 13, additionally comprising a carrier molecule.

15. An immunogen as claimed in claim 14, wherein the carrier molecule is selected from Protein D or Hepatitis B core antigen.

16. An immunogen as claimed in claim 14 or 15, wherein the immunogen is a chemical conjugate of the peptide or mimotope, or wherein the immunogen is expressed as a fusion protein.

17. An immunogen as claimed in any one of claims 14 to 16, wherein the peptide or peptide mimotope is presented within the primary sequence of the carrier.

18. A vaccine for the treatment of allergy comprising an immunogen as claimed in any one of claims 14 to 17, further comprising an adjuvant.

19. A ligand which is capable of recognising the peptides as claimed in any one of claims 1 to 13.

20. A ligand as claimed in claim 19, wherein the ligand is selected from P14/23, P14/31 or P14/33; which are deposited as Budapest Treaty patent deposit at ECACC on Jan. 26, 2000 under Accession No.s 00012610, 00012611, 00012612 respectively.

21. A pharmaceutical composition comprising a ligand as claimed in claim 19.

22. A pharmaceutical composition comprising a ligand as claimed in claim 20.

23. A peptide as claimed in any one of claims 1 to 13 for use in medicine.

24. A vaccine as claimed in claim 18 for use in medicine.

25. An immunogen as claimed in any one of claims 14 to 17, for use in medicine.

26. Use of a peptide as claimed in any one of claims 1 to 13 in the manufacture of a medicament for the treatment or prevention of allergy.

27. A ligand which is capable of recognising a peptide as claimed in any one of claims 1 to 13, for use in medicine.

28. Use of a ligand which is capable of recognising a peptide as claimed in any one of claims 1 to 13, in the manufacture of a medicament for the treatment of allergy.

29. Use of P14/23, P14/31 or P14/33; which are deposited as Budapest Treaty patent deposit at ECACC on Jan. 26, 2000 under Accession No.s

00012610, 00012611, 00012612 respectively, in the identification of mimotopes of P8.

30. A peptide which is capable of being recognised by P14/23, P14/31 or P14/33; which are deposited as Budapest Treaty patent deposit at ECACC on Jan. 26, 2000 under Accession No.s 00012610, 00012611, 00012612 respectively.

31. A vaccine comprising a peptide as claimed in claim 30.

32. A method of manufacturing a vaccine comprising the manufacture of an immunogen as claimed in any one of claims 14 to 17, and formulating the immunogen with an adjuvant.

33. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a peptide as claimed in any one of claims 1 to 13, to the patient.

34. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a vaccine as claimed in claim 24 or 31 to the patient.

35. A method of treating a patient suffering from or susceptible to allergy comprising administration of a pharmaceutical composition as claimed in any one of claims 21 or 22, to the patient.

L5 ANSWER 9 OF 12 USPATFULL on STN
2003:213269 Epitopes or mimotopes derived from the C-epsilon-3 or C-epsilon-4 domains of lgE, antagonists thereof, and their therapeutic uses.
Friede, Martin, Cardiff, CA, UNITED STATES
Mason, Sean, Cambridge, UNITED KINGDOM
Turnell, WIlliam Gordon, Cambridge, UNITED KINGDOM
Van Mechelen, Marcelle Paulette, Wagnelee, BELGIUM
Vinals y de Bassols, Carlotta, Brussels, BELGIUM
SmithKline Beecham Biologicals, s.a. (U.S. corporation)
US 2003147906 A1 20030807
APPLICATION: US 2002-322210 A1 20021218 (10)
PRIORITY: GB 1999-17144 19990721
GB 1999-18598 19990807
GB 1999-18599 19990807
GB 1999-18601 19990807
GB 1999-18604 19990807
GB 1999-18606 19990807
GB 1999-25618 19991029
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:

1. A peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is P5 (SEQ ID No. 1), or mimotope thereof.

2. A peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is P6 (SEQ ID No. 2), or mimotope thereof.

3. A peptide comprising an isolated surface exposed epitope of the region spanning Cε3 and Cε4 domains of IgE, wherein the peptide is P7 (SEQ ID No. 3), or mimotope thereof.

4. A peptide comprising an isolated surface exposed epitope of the Cε4 domain of IgE, wherein the peptide is P8 (SEQ ID No. 4), or mimotope thereof.

5. A peptide comprising an isolated surface exposed epitope of the Cε4 domain of IgE, wherein the peptide is P9 (SEQ ID No. 5), or mimotope thereof.

6. A peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is P200 (SEQ ID No. 6), or mimotope thereof.

7. A peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is P210 (SEQ ID No. 7), or mimotope thereof.

8. A peptide comprising an isolated surface exposed epitope of the Cε3 domain of IgE, wherein the peptide is 2-90N (SEQ ID No. 82), or mimotope thereof.

9. A peptide comprising an isolated surface exposed epitope of the Cε4 domain of IgE, wherein the peptide is 3-90N (SEQ ID No. 83), or mimotope thereof.

10. A peptide comprising an isolated surface exposed epitope of the Cε4 domain of IgE, wherein the peptide is 4-90N (SEQ ID No. 84), or mimotope thereof.

11. A mimotope as claimed in any one of claims 1 to 10 wherein the mimotope is a peptide.

12. A peptide as claimed in claim 4, wherein the mimotope of P8 is a peptide of the general formula: P, X_1, X_2, P, X_3, X_4, X_5, X_6, X_5, X_5 wherein; X_1 is an amino acid selected from E, D, N, or Q; X_2 is an amino acid selected from W, Y, or F; X_3 is an amino acid selected from G or A, X_4 is an amino acid selected from S, T or M; X_5 is an amino acid selected from R or K; and X_6 is an amino acid selected from D or E.

13. A peptide as claimed in claim 12, wherein the mimotope of P8 is a peptide of the general formula P, X_1, X_2, P, G, X_4, R, D, X_5, X_5 wherein; X_1 is an amino acid selected from E, D, N, or Q; X_2 is an amino acid selected from W, Y, or F; X_4 is an amino acid selected from S, T or M; X_5 is an amino acid selected from R or K; and X_6 is an amino acid selected from D or E.

14. An immunogen for the treatment of allergy comprising a peptide or mimotope as claimed in any one of claims 1 to 13, additionally comprising a carrier molecule.

15. An immunogen as claimed in claim 14, wherein the carrier molecule is selected from Protein D or Hepatitis B core antigen.

16. An immunogen as claimed in claim 14 or 15, wherein the immunogen is a chemical conjugate of the peptide or mimotope, or wherein the immunogen is expressed as a fusion protein.

17. An immunogen as claimed in any one of claims 14 to 16, wherein the peptide or peptide mimotope is presented within the primary sequence of the carrier.

18. A vaccine for the treatment of allergy comprising an immunogen as claimed in any one of claims 14 to 17, further comprising an adjuvant.

19. A ligand which is capable of recognising the peptides as claimed in any one of claims 1 to 13.

20. A ligand as claimed in claim 19, wherein the ligand is selected from P14/23, P14/31 or P14/33; which are deposited as Budapest Treaty patent deposit at ECACC on 26/1/00 under Accession No.s 00012610, 00012611, 00012612 respectively.

21. A pharmaceutical composition comprising a ligand as claimed in claim 19.

22. A pharmaceutical composition comprising a ligand as claimed in claim 20.

23. A peptide as claimed in any one of claims 1 to 13 for use in medicine.

24. A vaccine as claimed in claim 18 for use in medicine.

25. An immunogen as claimed in any one of claims 14 to 17, for use in medicine.

26. Use of a peptide as claimed in any one of claims 1 to 13 in the manufacture of a medicament for the treatment or prevention of allergy.

27. A ligand which is capable of recognising a peptide as claimed in any one of claims 1 to 13, for use in medicine.

28. Use of a ligand which is capable of recognising a peptide as claimed in any one of claims 1 to 13, in the manufacture of a medicament for the treatment of allergy.

29. Use of P14/23, P14/31 or P14/33; which are deposited as Budapest

Treaty patent deposit at ECACC on 26/1/00 under Accession No.s 00012610, 00012611, 00012612 respectively, in the identification of mimotopes of P8.

30. A peptide which is capable of being recognised by P14/23, P14/31 or P14/33; which are deposited as Budapest Treaty patent deposit at ECACC on 26/1/00 under Accession No.s 00012610, 00012611, 00012612 respectively.

31. A vaccine comprising a peptide as claimed in claim 30.

32. A method of manufacturing a vaccine comprising the manufacture of an immunogen as claimed in any one of claims 14 to 17, and formulating the immunogen with an adjuvant.

33. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a peptide as claimed in any one of claims 1 to 13, to the patient.

34. A method for treating a patient suffering from or susceptible to allergy, comprising the administration of a vaccine as claimed in claim 24 or 31 to the patient.

35. A method of treating a patient suffering from or susceptible to allergy comprising administration of a pharmaceutical composition as claimed in any one of claims 21 or 22, to the patient.

L5 ANSWER 10 OF 12 USPATFULL on STN
2003:123086 Vaccine adjuvants.
Friede, Martin, Court St Etienne, BELGIUM
Hermand, Philippe, Court St Etienne, BELGIUM
SmithKline Beechman Biologicals s.a., Rixensart, BELGIUM (non-U.S. corporation)
US 6558670 B1 20030506
APPLICATION: US 1999-301829 19990429 (9)
PRIORITY: BE 1999-8885 19990419
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. An immunogenic composition comprising a saponin an immunostimulatory oligonucleotide comprising an unmethylated CG dinucleotide and a tumor-associated antigen.

2. An immunogenic composition as claimed in claim 1 wherein said saponin is QS21.

3. An immunogenic composition as claimed in claim 1 wherein said immunostimulatory oligonucleotide comprises a sequence of XXCGYY, wherein X is a purine and Y is a pyrimidine.

4. An immunogenic composition as claimed in claim 1 wherein said immunostimulatory oligonucleotide is selected from the group comprising: TCC ATG ACG TTC CTG ACG TT (SEQ ID NO:1); TCT CCC AGC GTG CGC CAT (SEQ ID NO:2); ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG (SEQ ID NO:3).

5. A method of treatment of an individual susceptible to or suffering from a disease by the administration to an individual an immunogenic composition as claimed in any of claims 1 to 4.

L5 ANSWER 11 OF 12 USPATFULL on STN
2003:105857 Method to enhance an immune response of nucleic acid vaccination.
Dalemans, Wilfried, Hoegaarden, BELGIUM
Mechelen, Marcelle Van, Wagnelee, BELGIUM
Bruck, Claudine, Rixensart, BELGIUM
Friede, Martin, Farnham, UNITED KINGDOM
SmithKline Beecham Biologicals, s.a. (non-U.S. corporation)
US 2003072768 A1 20030417
APPLICATION: US 2002-292136 A1 20021112 (10)
PRIORITY: GB 1997-26555 19971216
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. A method to enhance an immune response of nucleic acid vaccination by simultaneous administration of: (i) a polynucleotide encoding for a polypeptide of interest; and (ii) the polypeptide of interest.

2. The method of claim 1 wherein the nucleic acid is DNA.

3. The method of claim 1 wherein the nucleic acid is RNA.

4. The method of claim 2 wherein the DNA and protein are admixed.

5. The method of claim 1 wherein the polypeptide is administered 0-10 days after the polynucleotide.

6. The method of claim 5 wherein the polypeptide is administered within 3-7 days after the polynucleotide.

7. The method of claim 1 wherein the polypeptide is presented in a delayed release formulation and administered at the same time as the polynucleotide.

8. A method to enhance an immune response of polypeptide vaccination by simultaneous administration of: (i) a nucleic acid encoding for a polypeptide of interest; and (ii) the polypeptide of interest.

9. The method of claim 8 wherein the nucleic acid is DNA.

10. The method of claim 8 wherein the nucleic acid is RNA.

11. The method of claim 9 wherein the DNA and protein are admixed.

12. The method of claim 8 wherein the polypeptide is presented in a delayed release formulation and administered at the same time as the polynucleotide.

13. The method of claim 8 wherein the immune response is a Th1 response.

14. A pharmaceutical composition comprising DNA+polypeptide, wherein the DNA encodes the polypeptide of interest and wherein the ratio of DNA:Polypeptide is from 1000:1 to 1:1 (w/w).

15. The pharmaceutical composition of claim 14 wherein the polypeptide is presented in a delayed release formulation.

16. The method of claim 15 wherein the polypeptide is coated with a biodegradable polymer comprising poly(capro-lactone) or poly(lactide-co-glycolide).

17. A method to prepare a pharmaceutical formulation according to claim 14, which method comprises: purifying a polynucleotide encoding for a polypeptide of interest; purifying a polypeptide of interest; and admixing the combination thereof.

18. A method to prepare a pharmaceutical formulation according to claim 15, which method comprises: purifying a polynucleotide encoding for a polypeptide of interest; purifying a polypeptide of interest; encapsulating the polypeptide of interest in a delayed release formulation; and admixing the combination thereof.

19. A vaccine comprising DNA+polypeptide, wherein the DNA encodes the polypeptide of interest, and wherein the ratio of DNA:Polypeptide is from 1000:1 to 1:1 (w/w).

20. The vaccine of claim 19 wherein the polypeptide is presented in a delayed release formulation.

21. The vaccine according to claim 19 comprising DNA+polypeptide and a suitable adjuvant.

22. The vaccine according to claim 20 comprising DNA+polypeptide presented in a delayed release formulation and a suitable adjuvant.

23. The use of DNA+polypeptide admixed together in the manufacture of a composition for use in enhancing the immune response of a mammal.

24. The use of DNA+polypeptide wherein the polypeptide is presented in a delayed release formulation admixed together in the manufacture of a composition for use in enhancing the immune response of a mammal.

L5 ANSWER 12 OF 12 USPATFULL on STN
2002:346653 Method to enhance an immune response of nucleic acid vaccination.
Dalemans, Wilfried, Hoegaarden, BELGIUM
Van Mechelen, Marcelle, Wagnelee, BELGIUM
Bruck, Claudine, Rixensart, BELGIUM

Friede, Martin, Farnham, UNITED KINGDOM
SmithKline Beecham Biologicals, S.A., Rixensart, BELGIUM (non-U.S. corporation)
US 6500432 B1 20021231
WO 9930733 19990624
APPLICATION: US 2000-581368 20000612 (9)
WO 1998-EP8152 19981211
PRIORITY: GB 1997-26555 19971216
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. A method for enhancing an immune response in an subject wherein the immune response is induced by administration of a polynucleotide, the method comprising the steps of: (1) administering a polynucleotide encoding a polypeptide; and (2) administering the polypeptide encoded by the polynucleotide administered in (1) wherein the polypeptide is administered 1-10 days after the polynucleotide is administered and wherein the method results in an increase in the immune response to the polypeptide as measured by one or more members selected from the group consisting of (i) total antibody titer, (ii) lymphoproliferation and (iii) cyctotoxic T cell level when compared to the immune response induced by administration of the polynucleotide in the absence of administration of the polypeptide.

2. The method of claim 1 wherein the polypeptide is administered 3-7 days after the polynucleotide is administered.

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FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006
E GARCON NATALIE/IN
L1 25 S E4-E7
L2 8 S L1 AND (CPG)
L3 17 S L1 NOT L2
E FRIEDE MARTIN/IN
L4 17 S E3
L5 12 S L4 NOT L1

=> s (WD1001 or WD1002 or WD1003 or WD1004 or WD1005 or WD1006 or WD1007)
2 WD1001
3 WD1002
10 WD1003
1 WD1004
1 WD1005
2 WD1006
1 WD1007
L6 12 (WD1001 OR WD1002 OR WD1003 OR WD1004 OR WD1005 OR WD1006 OR WD1007)

=> s l6 not l1
L7 11 L6 NOT L1

=> s l7 not l4
L8 11 L7 NOT L4

=> d l8,cbib,clm,kwic,l1

L8 ANSWER 11 OF 11 USPATFULL on STN
94:43107 Disk drive array with request fragmentation.
Jeffries, Kenneth L., 15807 Booth Cir., Leander, TX, United States 78641
Jones, Craig S., 12015 Scribe Dr., Austin, TX, United States 78759
US 5313585 19940517
APPLICATION: US 1991-810790 19911217 (7)
DOCUMENT TYPE: Utility; Granted.
CLM What is claimed is:
1. A method of operating one or more disk drives through a programmable disk drive controller which is interfaced through a bus to a host computer, comprising the steps of: (a) occasionally issuing access requests from said host computer to said disk drive controller; (b) maintaining a queue, in said disk drive controller, for said access requests; (c) analyzing any newly received access requests, in said disk drive controller, to ascertain whether said access request is atomic; and, if said access request is not atomic, then fragmenting said access request into smaller access requests, until no resulting access request is not atomic; (d) enqueuing atomic access requests into said queue,

together with execution-control markers which indicate groups of atomic operations which must be executed in strict sequence; (e) executing atomic access requests from said queue in an order which, except as constrained by said execution-control markers, is dynamically optimized in accordance with the instantaneous rotational phase of said disk drives.

2. The method of claim 1, wherein elements of said queue can include fence markers, and said controller, when processing said queue, accepts said fence markers as processing flow control commands.

3. The method of claim 1, wherein said controller processes said queue iteratively until all elements of said queue are atomic.

4. A method of operating one or more disk drives through a programmable disk drive controller which is interfaced through a bus to a host computer, comprising the steps of: (a) occasionally issuing access requests from said host computer to said disk drive controller; (b) maintaining a queue, in said disk drive controller, for said access requests; (c) analyzing any newly received access requests, in said disk drive controller, to ascertain whether said access request is atomic; and, if said access request is not atomic, then fragmenting said access request into smaller access requests, until no resulting access request is not atomic; (d) enqueuing atomic access requests into said queue, together with execution-control markers which indicate groups of atomic operations which must be executed in strict sequence; (e) executing atomic access requests from said queue in an order which, except as constrained by said execution-control markers, is dynamically optimized in accordance with the instantaneous rotational phase of said disk drives; (f) when said controller detects an error condition during execution of an access request, then inserting one or more error handling requests into said queue, and servicing said error handling requests before servicing any further access requests; (g) returning said redesignated access request to its original designation.

5. A method of redundant memory access request execution, comprising the steps of: (a) providing a queue for access requests to a memory; (b) redesignating an access request when said access request detects a defect in said memory; (c) inserting error handling requests in said queue prior to said redesignated access request; (d) executing said error handling requests; and (e) returning said redesignated access request to its original designation.

6. A disk drive controller, comprising, on a common circuit board: a microprocessor; random-access memory which is read/write accessible by said microprocessor; a bus interface circuit, connected to said microprocessor and to a system bus connector; multiple connections for cabling to multiple separate disk drives; wherein said microprocessor comprises: means for maintaining a queue, in said disk drive controller, for access requests received; means for analyzing any newly received access requests, in said disk drive controller, to ascertain whether said access request is atomic; and, if said access request is not atomic, then fragmenting said access request into smaller access requests, until no resulting access request is not atomic; means for enqueuing atomic access requests into said queue, together with execution-control markers which indicate groups of atomic operations which must be executed in strict sequence; means for executing atomic access requests from said queue in an order which, except as constrained by said execution-control markers, is dynamically optimized in accordance with the instantaneous rotational phase of said disk drives.

7. The controller of claim 6, wherein elements of said queue can include fence markers, and said controller, when processing said queue, accepts said fence markers as processing flow control commands.

8. The controller of claim 6, wherein said controller processes said queue iteratively until all elements of said queue are atomic.

9. The controller of claim 6, wherein each of said disk drives includes multiple rotating platters accessed by multiple respective movable read/write heads.

10. The controller of claim 6, wherein multiple ones of said disk drives include high-level integrated control electronics.

11. The controller of claim 6, wherein multiple ones of said disk drives include control electronics which implement a first remapping of defective sectors, and said first remapping is invisible to said controller.

12. The controller of claim 6, wherein multiple ones of said disk drives are IDE drives.

13. The controller of claim 6, wherein each of said disk drives includes multiple rotating platters accessed by multiple respective movable read/write heads, and wherein said movable heads of multiple ones of said disk drives move in synchrony to corresponding positions.

14. The controller of claim 6, wherein each of said disk drives includes multiple rotating platters accessed by multiple respective movable read/write heads, and wherein said movable heads of a first subset of ones of said disk drives move in synchrony to mutually corresponding positions, and said movable heads of a second subset of ones of said disk drives do not move in synchrony with heads of said drives of said first subset.

15. The controller of claim 6, wherein each of said drives includes multiple rotating platters accessed by multiple respective movable read/write heads, and wherein said controller is connected to access both first and second pluralities of said disk drives, and wherein said movable heads of a first subset of ones of said disk drives move in synchrony to mutually corresponding positions, and said movable heads of a second subset of ones of said disk drives do not move in synchrony with heads of said drives of said first subset, and said controller is connected to a system bus and presents an interface thereto whereby said drives of said first subset, but not said drives of said second subset, appear as a single composite disk drive.

16. The controller of claim 6, wherein each of said drives includes multiple rotating platters accessed by multiple respective movable read/write heads, and wherein said movable heads of a first subset of ones of said disk drives move in synchrony to mutually corresponding positions, and said movable heads of a second subset of ones of said disk drives do not move in synchrony with heads of said drives of said first subset.

17. The controller of claim 6, wherein said controller includes a microcontroller and a bus interface chip separate from said microcontroller.

18. The controller of claim 6, wherein said controller includes a bus-master interface chip.

19. The controller of claim 6, wherein said controller consists of multiple integrated circuits mounted on a single circuit board, and said controller includes multiple connections for cabling to ones of said drives.

20. The controller of claim 6, wherein said controller includes one or more connections which can each be connected to multiple ones of said drives in a daisy-chain configuration.

21. The controller of claim 6, wherein said controller includes analog signal interface connections for cabling to ones of said drives.

22. The controller of claim 6, wherein said disks each have integrated controllers which remap defective sectors invisibly to said disk drive controller.

23. The controller of claim 6, wherein said microprocessor is a microcontroller.

DETD . . . flag in the status register in AHA mode. Also used to clear the busy status in the status register in **WD1003** mode.

=> d his

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FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006

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            E GARCON NATALIE/IN
L1          25 S E4-E7
L2           8 S L1 AND (CPG)
L3          17 S L1 NOT L2
            E FRIEDE MARTIN/IN
L4          17 S E3
L5          12 S L4 NOT L1
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L6            12 S (WD1001 OR WD1002 OR WD1003 OR WD1004 OR WD1005 OR WD1006 OR
L7            11 S L6 NOT L1
L8            11 S L7 NOT L4

=> s l8 and (CpG)
          9147 CPG
L9            1 L8 AND (CPG)

=> d l9,cbib,clm,kwic

L9   ANSWER 1 OF 1  USPATFULL on STN
2005:36963 Vaccine.
    Dalemans, Wilfried L J., Hoegaarden, BELGIUM
    Gerard, Catherine Marie Ghislaine, Rhode Saint Genese, BELGIUM
    SmithKline Beecham Biologicals s.a. (non-U.S. corporation)
    US 2005031638 A1 20050210
    APPLICATION: US 2004-899771  A1  20040727 (10)
    PRIORITY: GB 1997-27262  19971224
    DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM    What is claimed is:
       1. A composition comprising an E6 or E7 protein or E6/E7 fusion protein
       from HPV optionally linked to an immunological fusion partner, and an
       immunomodulatory CpG oligonucleotide.

       2. A composition as claimed in claim 1 wherein the fusion partner is
       selected from the group; protein D or a fragment thereof from
       Heamophilus influenzae B, lipoprotein D or fragment thereof from
       Heamophilus influenzae B, NS1 or fragment thereof from Influenzae Virus,
       and LYTA or fragment thereof from Streptococcus Pneumoniae.

       3. A composition as claimed in claim 1 wherein the E6 or E7 proteins are
       derived from HPV16 or HPV18.

       4. A composition as claimed in claim 1 wherein the E7 protein is
       mutated.

       5. A composition as claimed in claim 1 wherein the E6 protein is
       mutated.

       6. A composition as claimed in claim 1 additionally comprising a
       hisitidine tag of at least 4 hisitidine residues.

       7. A composition as claimed herein comprising an additional HPV antigen.

       8. A composition as claimed herein where the immumodulatory CpG
       oligonucleotide comprises a hexamer motif: purine purine cytosine guaine
       pyrimidine pyrimidine.

       9. A composition as claimed herein wherein the immunomodulatory CpG
       oligonucleotide has two or more CpG motifs.

       10. A composition as claimed herein wherein the CpG oligonucleotide
       contains a phosphorothioate inter-nucleotide linkage.

       11. A composition as claimed herein wherein the CpG oligonucleotide is
       selected from the group:

       OLIGO 1:      TCC ATG ACG TTC CTG ACG TT

       OLIGO 2:      TCT CCC AGC GTG CGC CAT

       OLIGO 3:      ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG

       12. A composition as claimed herein for use in medicine.

       13. A method of inducing an immune response in a patient to an HPV
       antigen comprising administering a safe and effective amount of a
       composition as claimed herein.

       14. A method of preventing or treating HPV induced tumours in a patient
       comprising administering a safe and effective amount of a composition as
       claimed herein.

       15. A method of preparing a composition as claimed herein, comprising
       admixing an E6, E7 or E6/E7 fusion protein optionally linked to an
       immunological fusion partner, and an immunomodulatory CpG
       oligonucleotide.
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SUMM . . . or E6, E7 fusion protein from an HPV strain optionally linked with an immunological fusion partner and formulated with a **CpG** containing oligonucleotide into vaccines that find utility in the treatment or prophylaxis of human papilloma virus induced tumours or lesions.. . . that find utility in the treatment or prophylaxis of human papilloma induced tumours, wherein the vaccine is formulated with a **CpG** containing oligonucleotide as an adjuvant.

SUMM [0019] Immunomodulatory oligonucleotides contain unmethylated **CpG** dinucleotides ("**CpG**") and are known (WO 96/02555. EP 468520). **CpG** is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. Historically, it was observed that the DNA fraction of BCG. . .

SUMM . . . produce interferon γ and have cytolytic activity) and macrophages (Wooldrige et al Vol 89 (no. 8), 1977). Although other unmethylated **CpG** containing sequences not having this consensus sequence have now been shown to be immunomodulatory.

SUMM . . . an E6/E7 fusion protein optionally linked to an immunological fusion partner having T cell epitopes, and adjuvanted with an immunomodulatory **CpG** containing oligonucleotide.

SUMM [0025] Accordingly, the present invention in preferred embodiment provides compositions comprising an immunomodulatory **CpG** oligonucleotide and a fusion proteins comprising Protein D-E6 from HPV 16, Protein D-E7 from HPV 16 Protein D-E7 from HPV. . .

SUMM . . . the invention there is provided and E6 E7 fusion protein from HPV linked to an immunological fusion partner and a **CpG** immunomodulatory oligonucleotide.

SUMM [0052] The preferrred oligonucleotides preferably contain two or more **CpG** motifs separated by six or more nucleotides. The oligonucleotides of the present invention are typically deoxynucleotides. In a preferred embodiment. . .

SUMM [0054] The **CpG** oligonucleotides utilised in the present invention may be synthesized by any method known in the art (eg EP 468520). Conveniently,. . .

DETD [0239] The therapeutic potential of vaccine containing the PD1/3 E7 fusion protein and different **CpG** oligonucleotides were evaluated in the TC1 (E7 expressing tumour model.)

DETD [0249] 5) ProtD1/3 E7 HPV16+oligo 2/1758 (**WD1002**): TCT CCC AGC GTG CGC CAT

DETD [0256] As shown in FIGS. 1 and 2, in the groups of mice that received the antigen with a **CpG** oligonucleotide the mean tumour growth remained very low and very similar between groups, reflecting that the tumour growth either was. . .

DETD [0258] The mean tumour growth/group of mice vaccinated with PD1/3 E7+ the **CpG** oligos are quite similar and analysis of the individual tumour growth showed that the **CpG** oligos induce prolonged complete tumour rejection.

DETD [0260] Both **CpG** (Oligo 2>oligo 1) induced complete tumour regression.

DETD [0264] On the contrary, lymph node cells from mice that received ProtD1/3 E7 in **CpG** oligos 1 and 2 showed a very good E7 specific proliferative response although almost no PD (whole) specific response could. . .

DETD . . . with irradiated TC1 when TC1 or peptide E7 pulsed EL4, were used as target cells, when mice immunised with PD1/3 E7+**CpG** oligo 2>1 (25-40% specific lysis) and not with oligos alone.

DETD . . . cells than on peptide E7 pulsed EL4 cells, but this is mostly observed in the groups of mice vaccinated with PD1/3E7+**CpG** oligos (2>1). In this experiment other formulations did not induce a CTL.

DETD [0276] 1.3 Materials and Methods

Component	Brand	Batch number	Concentration (mg/ml)	Buffer
ProtD1/3-E7		957/015	0.677	PBS 7.4
oligo **CpG** 1826	EuroGentec	**WD1001**	5	H_{20}
oligo **CpG**	EuroGentec	**WD1002**	5	H_{20}

DETD [0280] Formulations containing oligo alone without other adjuvant were prepared by addition of **CpG** to the diluted PrtD1/3-E7 in PBS pH 7.4.

DETD . . . vaccinations, 7 and 14 days after the tumor challenge, with 5>g ProtD 1/3 E7 HPV16 injected intra-footpad (100 µl: 50 µl/footpad)+/-**CpG** oligo; Oligo 1 (**WD1001**) as a phosphorothioate modified or the same Oligo (**WD1006**) but with phosphodiester linkage.

DETD [0304] 100% of the animals receiving the PD1/3 E7 protein+oligo **WD1006** develop a tumor at the concentrations tested

DETD [0305] All the groups of animals that received the E7 protein+**CpG** 1001 at a concentration ranging from 10 to 200 µg show tumor regression partial or complete (20-40%).

DETD [0306] The first concentration at which this therapeutic effect on tumor

regression is not fully obtained is E7+1 µg **CpG** oligo 1001.

DETD [0312] The results (FIG. 6) of the experiments show that therapeutic vaccination with **CpG** oligonucleotide and antigen as described herein, results in a reduction of tumour growth and can induce complete tumour regression.

DETD . . . tumor challenge, with 51 g ProtD 1/3 E7 HPV16 injected intra-footpad (100 µl: 50 µl/footpad) in the 2 presence of **CpG** oligonucleotide TCT CCC AGC GTG CGC CAT and two control adjuvants,

. . . E6 or E7 protein or E6/E7 fusion protein from HPV optionally linked to an immunological fusion partner, and an immunomodulatory **CpG** oligonucleotide.

8. A composition as claimed herein where the immumodulatory **CpG** oligonucleotide comprises a hexamer motif: purine purine cytosine guaine pyrimidine pyrimidine.

9. A composition as claimed herein wherein the immunomodulatory **CpG** oligonucleotide has two or more **CpG** motifs.

10. A composition as claimed herein wherein the **CpG** oligonucleotide contains a phosphorothioate inter-nucleotide linkage.

11. A composition as claimed herein wherein the **CpG** oligonucleotide is selected from the group:

OLIGO 1: TCC ATG ACG TTC CTG ACG TT

OLIGO 2: TCT CCC. . .

. . . herein, comprising admixing an E6, E7 or E6/E7 fusion protein optionally linked to an immunological fusion partner, and an immunomodulatory **CpG** oligonucleotide.

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=> d his

     (FILE 'HOME' ENTERED AT 00:18:42 ON 13 NOV 2006)

     FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006
                E GARCON NATALIE/IN
L1             25 S E4-E7
L2              8 S L1 AND (CPG)
L3             17 S L1 NOT L2
                E FRIEDE MARTIN/IN
L4             17 S E3
L5             12 S L4 NOT L1
L6             12 S (WD1001 OR WD1002 OR WD1003 OR WD1004 OR WD1005 OR WD1006 OR
L7             11 S L6 NOT L1
L8             11 S L7 NOT L4
L9              1 S L8 AND (CPG)

=> s (TCCATGACGTTCCTGACGTT)
L10           154 (TCCATGACGTTCCTGACGTT)

=> s l10 not l1
L11           154 L10 NOT L1

=> s l11 not l4
L12           154 L11 NOT L4

=> s l12 and (CpG)
           9147 CPG
L13           154 L12 AND (CPG)

=> s l13 and (immunostimulatory)
           5467 IMMUNOSTIMULATORY
L14           138 L13 AND (IMMUNOSTIMULATORY)

=> s l14 and ay<2000
        3009073 AY<2000
L15            12 L14 AND AY<2000

=> d l15,cbib,clm,12

L15  ANSWER 12 OF 12  USPATFULL on STN
1999:121537 Peptides capable of modulating inflammatory heart disease.
    Bachmaier, Kurt, Toronto, Canada
    Hessel, Andrew John, Toronto, Canada
    Neu, Nickolaus, Innsbruck, Austria
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Penninger, Josef Martin, Toronto, Canada
Amgen Canada Inc., Mississauga, Canada (non-U.S. corporation)
US 5962636 19991005
APPLICATION: US 1998-133774 19980812 (9)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. A peptide selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:16.

2. The peptide of claim 1 wherein the amino-terminal amino acid is acylated.

3. The peptide of claim 2 wherein an acetyl group is used for acylation.

4. A peptide selected from the group consisting of: SEQ ID NO:3 and SEQ ID NO:15.

5. The peptide of claim 4 wherein the amino-terminal amino acid is acylated.

6. The peptide of claim 5 wherein an acetyl group is used for acylation.

7. A vaccine to decrease inflammatory cardiomyopathy comprising a peptide, an adjuvant, and an excipient, wherein the peptide consists of any of SEQ ID NOS; 2, 3, 4, 5, 6, 7, 8, 9, 15, or 16.

=> d l15,kwic,12

L15 ANSWER 12 OF 12 USPATFULL on STN
AI US 1998-133774 19980812 (9)
SUMM The term "**CpG** oligodeoxynucleotide" refers to an oligodeoxynucleotide containing the internal motif "GACGTT". Preferably, the **CpG** oligodeoxynucleotide will be about 20 nucleotides in length, but may range from about 14 to 30 or more nucleotides in. . .
SUMM . . . a gene encoding inflammatory cardiomyopathy peptide or fragment thereof, or therapeutic cardiomyopathy peptide or fragment thereof, or to prepare a **CpG** oligodeoxynucleotide of the present invention, is to employ chemical synthesis using methods well known to the skilled artisan such as. . .
SUMM Certain bacterial DNA molecules purportedly can have **immunostimulatory** effects in vivo and in vitro (Davis et al., J. Immunol. , 160: 870 -876 [1998]). However, prior to the present invention, it was not known that certain oligodeoxynucleotides having a **CpG** motif (GACGTT) could be useful as adjuvants for vaccines.
SUMM **TCCATGACGTTCCTGACGTT** (SEQ ID NO:12)
DETD . . . cysteine rich outer membrane protein from Chlamydia trachomatis (de la Maza et al., Infect. Immun., 59: 1196-1201 [1991]) containing a **CpG** motif and referred to as a "**CpG** oligo" (SEQ ID NO:13), and its counterpart not containing the **CpG** motif, the "non-**CpG** oligo" (SEQ ID NO:14), were synthesized using standard phosphoramidite chemistry, and were phosphorothioate modified (Stein et al., supra; Caruthers et. . .
DETD . . . 50 micrograms of M7A-alpha peptide (SEQ ID NO:2) and about 10 nmol of the oligodeoxynucleotide of either SEQ ID NO:13 (**CpG** oligodeoxynucleotide) or SEQ ID NO:14 (non-**CpG** oligodeoxynucleotide), together with Freund's incomplete adjuvant. Negative control mice received about 10 nmol of the oligodeoxynucleotide of SEQ ID NO:13. .
DETD TABLE 2

Adjuvant	Peptide	Prevalence	Severity
CFA	M7A-alpha	5/5	3.8 ± 0.4
CPG	M7A-alpha	5/5	1.2 ± 0.4
non-**CpG**	M7A-alpha	1/5	1.0 ± 0.0
CpG	None	0/5	--

DETD Surprisingly, the **CpG** oligonucleotide plus M7A-alpha peptide induced inflammatory heart disease in the absence of Freund's complete adjuvant, indicating that this oligonucleotide, which contains the **CpG** motif, can serve as a potent immunostimulator. The oligonucleotide containing the non-**CpG** motif was hardly effective as an adjuvant. Other **CpG** oligodeoxynucleotides tested and found to be **immunostimulatory** include the oligos set forth in SEQ ID Nos:10-12 (see above).

DETD . . . - # the DNA
#outer membrane protein fromeine rich
Chlamydia trachomatis containing a C - #pG motif and referred to as **CpG** oligo.
- <400> SEQUENCE: 13
20 ttgg
- <210> SEQ ID NO 14
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Chlamydia trachomatis
<220> FEATURE:
#from the DNANFORMATION: An oligonucleotide derived
#outer membrane protein fromeine rich
Chlamydia trachomatis which does not - # contain the **CpG** motif and referred to as a non-**CpG** oligo.
- <400> SEQUENCE: 14
20 ttgg
- <210> SEQ ID NO 15
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Human
- <400> SEQUENCE: 15
-. . .

=> d l15,cbib,clm,kwic,1-12

L15 ANSWER 1 OF 12 USPATFULL on STN
2006:127412 Compositions of **CPG** and saponin adjuvants and uses thereof.
Kensil, Charlotte A., Milford, MA, UNITED STATES
Antigenics Inc., Lexington, MA, UNITED STATES (U.S. corporation)
US 7049302 B1 20060523
APPLICATION: US 1999-369941 19990806 (9)
PRIORITY: US 1999-128608P 19990408 (60)
US 1998-95913P 19980810 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide is not a part of a DNA vaccine vector, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

2. The immune adjuvant composition as claimed in claim 1, wherein the saponin comprises a substantially pure saponin.

3. The immune adjuvant composition as claimed in claim 2, wherein the substantially pure saponin is QS-7, QS-17, QS-18, or QS-21.

4. The immune adjuvant composition as claimed in claim 3, wherein the substantially pure saponin is QS-21.

5. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises more than one unmethylated **CpG** dinucleotide.

6. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester.

7. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

8. The immune adjuvant composition as claimed in claim 1, wherein the **immunostimulatory** oligonucleotide comprises a **CpG** motif having the formula 5'X_{1CGX23}', wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

9. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1).

10. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the saponin is substantially pure, and the saponin is QS-7, QS-17 or QS-18, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

11. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 10 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

12. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

13. The immune adjuvant composition as claimed in claim 12, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

14. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 12 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

15. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 13 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

16. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTFCGCCAT (SEQ ID NO:1), and, wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

17. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 16 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

18. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide comprises **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2), and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

19. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 18 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

20. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide is 4-40 bases in length, and wherein

the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

21. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 20 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

22. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin (i) is derived from Quillaja saponaria and (ii) is a chemically modified saponin; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

23. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 22 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

24. The composition of claim 1, wherein the saponin is a chemically modified saponin.

25. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

26. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 1 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

27. The method as claimed in any of claims 14, 15, 17, 19, 21, 23, or 26, wherein the saponin comprises is a substantially pure saponin.

28. The method as claimed in claim 27, wherein the substantially pure saponin is QS-7, QS-17, QS-18, or QS-21.

29. The method as claimed in claim 28, wherein the substantially pure saponin is QS-21.

30. The method as claimed in any of claims 11, 14, 15, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises more than one unmethylated **CpG** dinucleotide.

31. The method as claimed in any of claims 11, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester.

32. The method as claimed in any of claims 11, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

33. The method as claimed in any of claims 11, 14, 15, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises a **CpG** motif having the formula 5'X_{1CGX23}', wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

34. The method as claimed in any of claims 11, 14, 15, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

35. The method as claimed in any of claims 11, 14, 15, 21, 19, 21, 23, or 26, wherein the individual is an animal.

36. The method as claimed in claim 35, wherein the animal is a mammal.

37. The method as claimed in any of claims 11, 14, 15, 21, 19, 21, 23, or 26, wherein the individual is a human.

38. A vaccine composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide; and (c) a nucleic acid molecule comprising a nucleotide sequence encoding an antigen, wherein the nucleotide sequence is operatively linked to a promoter, wherein the **immunostimulatory** oligonucleotide is not a part of the nucleic acid molecule comprising the nucleotide sequence encoding the antigen.

39. The vaccine composition as claimed in claim 38, wherein the saponin is a substantially pure saponin.

40. The vaccine composition as claimed in claim 39, wherein the substantially pure saponin is QS-7, QS-17, QS-18, or QS-21.

41. The vaccine composition as claimed in claim 40, wherein the substantially pure saponin is QS-21.

42. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises more than one unmethylated **CpG** dinucleotide.

43. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester.

44. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

45. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises a **CpG** motif having the formula $5'X_{1CGX23}'$, wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

46. The vaccine composition as claimed in claim 38 or 41, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

47. The method of any of claims 11, 17, 19, 23, or 26, wherein the nucleic acid molecule encoding the antigen is administered to the individual concurrently with the immune adjuvant composition.

48. The method of any of claims 14, 15, or 21, wherein the nucleic acid molecule encoding the antigen is administered to the individual concurrently with the immune adjuvant composition.

49. The method as claimed in any of claims 14, 15, 21, 23, and 26, wherein the saponin is substantially pure, wherein the saponin is QS-21, and wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

50. The immune adjuvant composition as claimed in claim 12 or 20, wherein the saponin is chemically modified.

51. The immune adjuvant composition as claimed in claim 12, 20 or 22, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

52. The immune adjuvant composition as claimed in claim 12 or 22, wherein the saponin is substantially pure.

53. The immune adjuvant composition as claimed in claim 52, wherein the saponin is QS-21.

54. The immune adjuvant composition as claimed in claim 53, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

55. The immune adjuvant composition as claimed in claim 20, wherein the saponin is substantially pure.

56. The immune adjuvant composition as claimed in claim 55, wherein the saponin is QS-21.

57. The immune adjuvant composition as claimed in claim 20 or 56, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester.

58. The immune adjuvant composition as claimed in claim 56, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

59. The immune adjuvant composition as claimed in claim 16 or 18, wherein the saponin is substantially pure.

60. The immune adjuvant composition as claimed in claim 59, wherein the saponin is QS-21.

TI Compositions of **CPG** and saponin adjuvants and uses thereof

AI US 1999-369941 19990806 (9)

AB Vaccine compositions of **immunostimulatory** oligonucleotides and saponin adjuvants and antigens and the use thereof for stimulating immunity, enhancing cell-mediated immunity, and enhancing antibody production are disclosed. Also described are immune adjuvant compositions comprising **immunostimulatory** oligonucleotides and saponin adjuvants, as well as methods for increasing an immune response using the same.

SUMM Recently, oligonucleotides containing the unmethylated cytosine-guanine ("**CpG**") dinucleotide in a particular sequence context or motif have been shown to be potent stimulators of several types of immune cells in vitro. (Weiner, et al., Proc. Natl. Acad. Sci. 94:10833 (1997).) An **immunostimulatory** oligonucleotide comprising an unmethylated **CpG** motif is an dinucleotide within the oligonucleotide that consistently triggers an **immunostimulatory** response and release of cytokines. **CpG** motifs can stimulate monocytes, macrophages, and dendritic cells that can produce several cytokines, including the T helper 1 ("Th 1"). . . . et al., J. Exp. Med. 186:1623 (1997).) Klinman, et al., have shown that a DNA motif consisting of an unmethylated **CpG** dinucleotide flanked by two 5' purines (GpA or ApA) and two 3' pyrimidines (TpC or TpT) optimally stimulated B cells. . . et al., the contents of which are incorporated herein by reference, discovered that nucleic acids containing at least one unmethylated **CpG** dinucleotide may affect the immune response of a subject (Davis, et al., WO 98/40100, PCT/US98/04703).

SUMM . . . adjuvants may be potentially incorporated in future human vaccines. Surprisingly, a combination of an oligonucleotide comprising at least one unmethylated **CpG** dinucleotide and a saponin adjuvant was found to be a powerful stimulator of cell-mediated immunity compared to either adjuvant alone. Antibody titers (antigen-specific) in response to vaccination were significantly higher for vaccines comprising a **CpG**-containing oligonucleotide/saponin adjuvant combination compared to either saponin or **CpG** alone and represented a positive synergistic adjuvant effect. Together, these results establish that an immune adjuvant composition comprising an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide and a saponin adjuvant is a candidate adjuvant composition for vaccines to induce immunity. Accordingly, the present invention provides novel vaccine compositions which comprise an **immunostimulatory** oligonucleotide, a saponin adjuvant, and an antigen. Methods for increasing the immune response to an antigen by administrating the inventive. . .

DRWD FIG. 1 depicts a graph showing the enhancement of a cell-mediated immune response by QS-21 and **CpG** oligonucleotide/QS-21 combination, as evidenced by the CTL induction.

DRWD FIG. 2 provides a graph showing the enhancement of a cell-mediated immune response by QS-21 and **CpG** oligonucleotide/QS-21 combination, as evidenced by the CTL induction.

DRWD . . . graph of IgG1 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and for combinations of QS-21 and **CpG** oligonucleotide in mouse sera collected 21 days after a first immunization given on day 0.

DRWD . . . bar graph of IgG2a titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and **CpG** oligonucleotide in mouse sera collected 21 days after a first immunization given on day 0.

DRWD . . . bar graph of IgG3 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and **CpG** oligonucleotide in mouse sera collected 21 days after a first immunization given on day 0.

DRWD . . . bar graph of IgG1 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and **CpG** oligonucleotide in mouse sera collected 14 days after a

second immunization given 28 days after the first immunization.

DRWD . . . bar graph of IgG2a titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and **CpG** oligonucleotide in mouse sera collected 14 days after a second immunization given 28 days after the first immunization.

DRWD . . . bar graph of IgG3 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and **CpG** oligonucleotide in mouse sera collected 14 days after a second immunization given 28 days after the first immunization.

DETD The present invention may also employ **immunostimulatory** saponins isolated from other plant species. For example, a saponin from Dolichos lablab has been shown to be useful as. . .

DETD The term "**immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide" means an oligonucleotide that has been shown to activate the immune system. The **immunostimulatory** oligonucleotide may, preferably, comprise at least one unmethylated **CpG** dinucleotide. A "**CpG** motif" is a stretch of DNA comprising one or more **CpG** dinucleotides within a specified sequence. The oligonucleotide comprising the **CpG** motif may be as short as 4-40 base pairs in length. The **immunostimulatory** oligonucleotide containing the **CpG** motif may be a monomer or part of a multimer. Alternatively, the **CpG** motif may be a part of the sequence of a vector that also presents a DNA vaccine. It may be. . . double-stranded. It may be prepared synthetically or produced in large scale in plasmids. One embodiment of the invention covers the **immunostimulatory** oligonucleotide which contains a **CpG** motif having the formula 5'X_{1CGX23}', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine or adenine. In a preferred embodiment, the **CpG** motif comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1; also known as "1758") or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2; also known as "1826").

DETD DNA containing unmethylated **CpG** dinucleotide motifs in the context of certain flanking sequences has been found to be a potent stimulator of several types. . . (1996); Cowdrey, et al., J. Immunol. 156:4570 (1996); Krieg, et al., Nature 374:546 (1995).) Depending on the flanking sequences, certain **CpG** motifs may be more **immunostimulatory** for B cell or T cell responses, and preferentially stimulate certain species. When a humoral response is desired, preferred **immunostimulatory** oligonucleotides comprising an unmethylated **CpG** motif will be those that preferentially stimulate a B cell response. When cell-mediated immunity is desired, preferred **immunostimulatory** oligonucleotides comprising at least one unmethylated **CpG** dinucleotide will be those that stimulate secretion of cytokines known to facilitate a CD8+ T cell response.

DETD The **immunostimulatory** oligonucleotides of the invention may be chemically modified in a number of ways in order to stabilize the oligonucleotide against. . . of the oligonucleotide have been replaced with any number of non-traditional bases or chemical groups, such as phosphorothioate-modified nucleotides. The **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide may preferably be modified with at least one such phosphorothioate-modified nucleotide. Oligonucleotides with phosphorothioate-modified linkages may be prepared using. . .

DETD In a first aspect of the invention, an immune adjuvant composition comprising a saponin adjuvant and an **immunostimulatory** oligonucleotide may be administered. More preferably, such immune adjuvant composition may increase the immune response to an antigen in an. . . saponin adjuvant is QS-21. Alternatively, the immune adjuvant composition may comprise more than one substantially pure saponin adjuvant with the **immunostimulatory** oligonucleotide. In a further preferred embodiment, the saponin adjuvant may cover a chemically modified saponin adjuvant or a fraction thereof. . . at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin retains adjuvant activity. The **immunostimulatory** oligonucleotide, preferably, compries at least one unmethylated **CpG** dinucleotide. The **CpG** dinucleotide is preferably a monomer or multimer. Another preferred embodiment of the **CpG** motif is as a part of the sequence of a vector that also presents a DNA vaccine. Yet another embodiment of the immune adjuvant composition is directed to the **immunostimulatory** oligonucleotide, wherein the **immunostimulatory** oligonucleotide is modified. The particular modification may comprise at least one phosphorothioate-modified nucleotide. Further, the **immunostimulatory** oligonucleotide having at least one unmethylated **CpG** dinucleotide may comprise a **CpG** motif having the formula 5'X_{1CGX23}', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. The **CpG** motif may preferentially be TCTCCCAGCGTGCGCCAT [SEQ ID NO.:1] or **TCCATGACGTTCCTGACGTT** [SEQ ID NO.:2]

DETD . . . the antigen is administered comprising administering an

effective amount of an immune adjuvant composition comprising a saponin adjuvant and an **immunostimulatory** oligonucleotide further. Preferably, the saponin adjuvant is a saponin from Quillaja saponaria Molina. More preferably, the saponin adjuvant is a. . . saponaria Molina. The method may also embody an immune adjuvant composition comprising more than one substantially pure saponin adjuvant and **immunostimulatory** oligonucleotide. The substantially pure saponin adjuvant is preferably QS-7, QS-17, QS-18, or QS-21. Most preferably, the substantially pure saponin adjuvant. . . QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin retains adjuvant activity. In a preferred embodiment of the method, the **immunostimulatory** oligonucleotide comprises at least one unmethylated **CpG** dinucleotide. The **CpG** motif is preferably a monomer or a multimer. Another preferred embodiment of the method includes the **CpG** motif as a part of the sequence of a vector that presents a DNA vaccine. Yet another embodiment is directed to the method wherein the **immunostimulatory** oligonucleotide comprises at least one unmethylated **CpG** dinucleotide, and wherein furthermore, the **immunostimulatory** oligonucleotide may be chemically modified to stabilize the oligonucleotide against endogenous endonucleases. The modification may comprise at least one phosphorothioate-modified nucleotide. Further, the method may be directed, in part, to the **immunostimulatory** oligonucleotide having at least one unmethylated **CpG** dinucleotide comprising a **CpG** motif having the formula $5'X_{1CGX23}'$, wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. In another preferred method, the unmethylated **CpG** motif is TCTCCCATCGTGCGCCAT [SEQ ID NO.:1] or **TCCATGACGTTCCTGACGTT** [SEQ ID NO.:2]

DETD . . . response. A vaccine composition, according to the invention, would produce immunity against disease in individuals. The combination of saponin and **immunostimulatory** oligonucleotide of the present invention may be administered to an individual to enhance the immune response to any antigen. Preferably,. . .

DETD . . . the invention may enhance antibody production to an antigen in a positive synergistic manner. The synergistic adjuvant effect of the **immunostimulatory** oligonucleotide and the saponin adjuvant described herein may be shown in a number of ways. For example, a synergistic adjuvant. . .

DETD Accordingly, in a third aspect, the invention also encompasses a vaccine composition comprising a saponin adjuvant, an **immunostimulatory** oligonucleotide, and an antigen. The saponin adjuvant may be partially pure or substantially pure saponin from Quillaja saponaria Molina. The vaccine compositions may also comprise more than one partially pure or substantially pure saponin adjuvant, an **immunostimulatory** oligonucleotide further comprising at least one unmethylated **CpG** motif, and an antigen. Preferably, the partially pure saponin adjuvant comprises QS-7, QS-17, QS-18, and/or QS-21 and may comprise other retains adjuvant activity. Most preferably, the partially pure or substantially pure saponin adjuvant in the vaccine composition is QS-21. The **immunostimulatory** oligonucleotide may preferably comprise at least one unmethylated **CpG** dinucleotide. The **CpG** motif may preferably be a monomer or a multimer. Another preferred embodiment of the **CpG** motif is as a part of the sequence of a vector that also presents a DNA vaccine. Yet another embodiment of the vaccine composition described herein is directed to the **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide comprises a chemical modification. More particularly, the **immunostimulatory** oligonucleotide may be modified with at least one phosphorothioate-modified nucleotide. Further, the **immunostimulatory** oligonucleotide having at least one unmethylated **CpG** dinucleotide of the vaccine composition comprises a **CpG** motif having the formula $5'X_{1CGX23}'$, wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. The unmethylated **CpG** motif according to this aspect of the invention may preferentially comprise TCTCCCAGCGTGCGCCAT [SEQ ID NO.:1]or **TCCATGACGTTCCTGACGTT** [SEQ ID NO.:2]

DETD . . . an effective amount of a vaccine composition comprising an antigen, a partially pure or substantially pure saponin adjuvant, and an **immunostimulatory** oligonucleotide. The method also embodies a vaccine composition comprising more than one partially pure or substantially pure saponin adjuvant, an **immunostimulatory** oligonucleotide, and an antigen. Preferably, the partially pure saponin adjuvant comprises QS-7, QS-17, QS-18, and/or QS-21 and may comprise other. . . QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin retains adjuvant activity. Preferably, the method comprises administering an **immunostimulatory** oligonucleotide which further comprises at least one unmethylated **CpG** dinucleotide. The **CpG** dinucleotide therein is a monomer or a multimer. Another preferred embodiment of the method includes the **CpG** motif as a part of the

sequence of a vector that also presents a DNA vaccine. Yet another embodiment of the method disclosed herein is directed to the **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide may be chemically modified to increase its stability to endogenous endonucleases. Such a modification may comprise at least one phosphorothioate-modified nucleotide. Further, the **immunostimulatory** oligonucleotide having at least one unmethylated **CpG** dinucleotide may comprise a **CpG** motif having the formula 5'X_{1CGX23}', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. In another preferred embodiment, the unmethylated **CpG** motif is TCTCCCAGCGTGCGCCAT [SEQ ID NO.:1] or **TCCATGACGTTCCTGACGTT** [SEQ ID NO.:2]

DETD A well-established animal model was used to assess whether formulations of **CpG** oligonucleotide and QS-21 together could function as an immune adjuvant. In brief, experiments were set up to compare QS-21 to the recently reported adjuvant **CpG** motif. A **CpG** sequence (e.g., 1758), reported to serve as an adjuvant for a B-cell lymphoma idiotype-KLH vaccine in mice, was selected. One experiment evaluated whether the **CpG** motif, alone or in combination with QS-21, can serve as an adjuvant for a subunit vaccine, e.g., OVA, in mice in inducing CTL responses. This work included a dose range experiment with **CpG** to determine the optimum dose.

DETD In addition to comparing **CpG** and QS-21 as adjuvants, a second experiment combining **CpG** oligonucleotide with suboptimal doses of QS-21 (e.g., 1.25 μg) was conducted to assess whether **CpG** oligonucleotide can affect the adjuvant effect of QS-21.

DETD Also, an experiment was performed to determine whether the **CpG** and QS-21 combination could enhance antibody production, specifically the isotype profile of a antigen-specific antibody response.

DETD Finally, a series of experiments were performed to determine whether a combination of **CpG** oligonucleotide and saponin would enhance antibody production in a positive synergistic manner. This work used vaccine formulations of pneumococcal Type 14 polysaccharide and QS-21 and **CpG** oligonucleotide and evaluated specific antibody titers harvested from mice on days 21 and 42 after immunization on days 0 and 28. Another **CPG** sequence (e.g., 1826), reported to serve as an adjuvant for hen egg lysozyme in mice, was selected.

DETD . . . experiments were done using materials from the following suppliers: OVA, Grade VI (Sigma); pneumococcal Type 14 polysaccharide (ATCC); QS-21 (Aquila); **CpG** oligonucleotides included the phosphorothiate-modified sequence 1758 TCTCCCAGCGTGCGCCAT [SEQ ID NO.:1] and phosphorothiate-modified sequence 1826 **TCCATGACGTTCCTGACGTT** [SEQ ID NO.:2] (Life Technologies (Gibco)).

DETD CTL Induced by OS-21 and **CpG**/OS-21

DETD . . . 25 ng OVA antigen plus the indicated doses of adjuvant in a total volume of 0.2 ml phosphate-buffered saline. The **CpG** motif used in this experiment was a phosphorothioate-modified oligonucleotide 1758 with a sequence of TCTCCCAGCGTGCGCCAT [SEQ ID NO.:1] (Weiner, et. . .

DETD The results, as shown in FIG. 1, indicate that no lysis was observed in the absence of adjuvant, with any **CpG** dose, or with 1.25 μg of QS-21 (suboptimal dose). However, the suboptimal dose of QS-21, in combination with **CpG**, induced significant CTL. The results show a substantial adjuvant effect at doses that are normally not expected to produce such an adjuvant effect. This positive synergistic effect was most notable at the higher dose of **CpG** (50 μg). The adjuvant effect was comparable to that achieved with the optimal 10 μg QS-21 control.

DETD CTL Induced by OS-21 and **CpG**/OS-21

DETD As evident from the results in FIG. 2, no lysis was observed in the absence of adjuvant, with any **CpG** dose, or with 1.25 μg of QS-21 (suboptimal dose). However, the suboptimal dose of QS-21, in combination with **CpG**, induced significant CTL (comparable to the optimal 10 μg QS-21 control). The results illustrate the positive synergism between the **CpG** and the QS-21 that was unexpected at a suboptimal dose.

DETD . . . not detectable in any groups except for the combination of 10 μg QS-21 (optimal dose) with 10 or 50 μg **CpG** and the combination of 1.25 μg QS-21 (suboptimal dose) with 50 μg **CpG**. IgG2a was not detected with any **CpG** dose used alone, with any QS-21 dose used alone, or in the unadjuvanted group.

DETD Antibody Induced by OS-21 and OS-21/**CpG** to Pneumococcal Polysaccharide Antigen

DETD . . . pneumococcal Type 14 polysaccharide plus the indicated doses of adjuvant in a total volume of 0.2 ml phosphate-buffered saline. The **immunostimulatory** motif **CpG** used in this experiment was a phosphorothioate-modified oligonucleotide 1826 with a sequence of **TCCATGACGTTCCTGACGTT** [SEQ ID NO.:2] (Chu, et al., Exp. Med. 186:1623-1631 (1997)). QS-21 was used at a dose of 1.25 μg or 10 μg. **CpG** ODN 1826 was used at a dose of only 10 μg.

DETD . . . mice in each group. After a single immunization, IgG1 titers were 66 fold higher for the 10 μg QS-21/10 μg **CpG** combination than for QS-21 alone and were 43 fold higher than for **CpG** alone (FIG. 4). IgG2a titers were 11 fold higher for the 10 μg QS-21/**CpG** combination than for either QS-21 alone or **CpG** alone (FIG. 5). IgG3 titers were 85 fold higher for the 10 μg QS-21/**CpG** combination than for QS-21 alone and were 95 fold higher than for **CpG** alone (FIG. 6).

DETD After two immunizations, IgG1 titers were 46 fold higher for the 10 μg QS-21/**CpG** combination than for QS-21 alone and were 444 fold higher than for **CpG** alone (FIG. 7). IgG2a titers were 476 fold higher for the 10 μg QS-21/**CpG** combination than for QS-21 alone and were 127 fold higher than for **CpG** alone (FIG. 5). IgG3 titers were 67 fold higher for the 10 μg QS-21/**CpG** combination than for QS-21 alone and were 243 fold higher than for **CpG** alone (FIG. 9). The enhancement of these titers shows that this is a positive synergistic effect and is not simply. . . effect of combining these two adjuvants. In addition, the combination of low doses of QS-21 (1.25 μg) with 10 μg **CpG** also produced IgG1 and IgG3 titers after two immunizations that were higher than those produced by either 1.25 μg QS-21 alone, 10 μg QS-21 alone, or 10 μg **CpG** alone.

. . . composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide is not a part of a DNA vaccine vector, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

5. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises more than one unmethylated **CpG** dinucleotide.

6. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate,. . .

7. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

8. The immune adjuvant composition as claimed in claim 1, wherein the **immunostimulatory** oligonucleotide comprises a **CpG** motif having the formula 5'X_1CG$X_2$3', wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

9. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1).

. . . composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the saponin is substantially pure, and the saponin is QS-7, QS-17 or QS-18, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

. . . composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

13. The immune adjuvant composition as claimed in claim 12, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

. . . composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTFCGCCAT (SEQ ID NO:1), and, wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic

adjuvant effect.

. . . composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide comprises **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2), and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

. . . composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide is 4-40 bases in length, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

. . . activity, wherein the saponin (i) is derived from Quillaja saponaria and (ii) is a chemically modified saponin; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

25. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

. . . 30. The method as claimed in any of claims 11, 14, 15, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises more than one unmethylated **CpG** dinucleotide.

31. The method as claimed in any of claims 11, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate,. . .
32. The method as claimed in any of claims 11, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

. . . 33. The method as claimed in any of claims 11, 14, 15, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises a **CpG** motif having the formula $5'X_1CGX_2 3'$, wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

34. The method as claimed in any of claims 11, 14, 15, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

. . . vaccine composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide; and (c) a nucleic acid molecule comprising a nucleotide sequence encoding an antigen, wherein the nucleotide sequence is operatively linked to a promoter, wherein the **immunostimulatory** oligonucleotide is not a part of the nucleic acid molecule comprising the nucleotide sequence encoding the antigen.

42. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises more than one unmethylated **CpG** dinucleotide.

43. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate,. . .
44. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

45. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises a **CpG** motif having the formula $5'X_1CGX_2 3'$, wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

46. The vaccine composition as claimed in claim 38 or 41, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID

NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

. . . 14, 15, 21, 23, and 26, wherein the saponin is substantially pure, wherein the saponin is QS-21, and wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

51. The immune adjuvant composition as claimed in claim 12, 20 or 22, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

54. The immune adjuvant composition as claimed in claim 53, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

57. The immune adjuvant composition as claimed in claim 20 or 56, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate,. . .
58. The immune adjuvant composition as claimed in claim 56, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

L15 ANSWER 2 OF 12 USPATFULL on STN
2004:241962 Use of penetration enhancers and barrier disruption agents to enhance the transcutaneous immune response.
Glenn, Gregory M., Cabin John, MD, United States
Alving, Carl R., Bethesda, MD, United States
The United States of America as represented by the Secretary of the Army, Washington, DC, United States (U.S. government)
US 6797276 B1 20040928
APPLICATION: US 1999-257188 19990225 (9)
PRIORITY: US 1998-86251P 19980521 (60)
US 1998-75856P 19980225 (60)
US 1998-75850P 19980225 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. A method for inducing an antigen specific immune response in a subject comprising: a. pretreating an are of the skin of said subject; and b. applying a formulation to said pretreated area, wherein said formulation comprises: 1) at least one antigen sufficient to induce an antigen-specific immune response against a pathogen, 2) at least one adjuvant present in an amount effective to induce said immune response to said at least one antigen; and, 3) a pharmaceutically acceptable carrier, wherein said pretreating enhances skin penetration by said formulation and thereby induces said immune response, wherein said pretreating is selected from the group consisting of direct application to said skin, rubbing, swabbing, applying a depilatory agent, applying a keratinolytic formulation, shaving, tape stripping, abrading and a combination thereof.

2. The method of claim 1, wherein said swabbing comprises a swab comprising a material selected from the group consisting of cotton, nylon, wool and combinations thereof.

3. The method of claim 2, wherein said swab further comprises an alcohol, a composition comprising an alcohol, acetone, a composition comprising acetone, a detergent or a detergent solution.

4. The method of claim 1 wherein said pretreating comprises applying a detergent or a detergent solution to said pretreated area.

5. The method of claim 1, wherein said antigen is derived from a pathogen selected from the group consisting of virus, bacteria, fungus and parasite.

6. The method of claim 1, wherein said antigen is derived from an influenza virus.

7. The method of claim 6, wherein said antigen is hemaglutinin A.

8. The method of claim 1, wherein said antigen is derived from a bacteria.

9. The method of claim 8, wherein said antigen is E. coli heat-labile entertoxin (LT).

10. The method of claim 1, further comprising a carrier, wherein said carrier is a patch.

11. The method of claim 10, wherein said patch is selected from the group consisting of an occlusive dressing, a nonocclusive dressing, a hydrogel dressing and a reservoir dressing.

AI **US 1999-257188 19990225 (9)**

DETD . . . immune responses (Medzhitov and Janeway, 1997). These structures are called pathogen-associated molecular patterns (PAMPs) and include lipopolysaccharides, teichoic acids, unmethylated **CpG** motifs, double stranded RNA and mannins, for example.

DETD . . . failed to induce a detectable rise in the anti-DT titers, In contrast, addition of a DNA sequence containing an unmethylated **CpG** dinucleotide flanked by two 5' purines and two 3' pyrimidines (SEQ ID NO:2; **immunostimulatory** CpG1: **TCCATGACGTTCCTGACGTT**) resulted in a detectable increase in the serum anti-DT IgG titer in 5 of 5 animals Thus it appears that. . .

DETD . . . as a readout. The results are shown in Table 7B: Co-administration of DT and a DNA sequence containing an unmethylated **CpG** dinucleotide flanked by two 5' purines and two 3' pyrimidines (SEQ ID NO:2) resulted in a detectable increase in the. . .

DETD . . . isopropanol. After the alcohol had evaporated (approximately 5 minutes), 100 µl of phosphate buffered saline (PBS) containing 100 µg of **immunostimulatory** DNA (CpG1) and/or cholera toxin (CT) 100 µg was applied to the back with 100 µg of a soluble leishmanial. . .

DETD Co-administration of SLA and CpG1 (**immunostimulatory** DNA containing an unmethylated **CpG** dinucleotide flanked by two 5' purines and two 3' pyrimidines--SEQ ID NO:2) or CT resulted in a detectable increase in. .

DETD

TABLE 11

Synergy between **immunostimulatory** DNA and ADP ribosylating exotoxin (CT) as adjuvants when applied to the skin
proliferation (cpm) 3-H incorporation
in vitro to antigens
substances. . .

L15 ANSWER 3 OF 12 USPATFULL on STN

2003:309071 Method of treating cancer using **immunostimulatory** oligonucleotides

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US 6653292 B1 20031125
APPLICATION: US 1999-337619 19990621 (9)
DOCUMENT TYPE: Utility; GRANTED.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A method for increasing the responsiveness of a cancer cell to a cancer therapy using an **immunostimulatory** nucleic acid as compared to the absence of the **immunostimulatory** nucleic acid, comprising: administering to a subject having a cancer an effective amount for increasing the responsiveness of a cancer cell to a cancer therapy of an **immunostimulatory** nucleic acid, comprising: 5'$X_{1X2CGX3X43}$' wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides, and wherein the sequence is not palindromic.

2. The method of claim 1, further comprising administering a chemotherapeutic agent.

3. The method of claim 1, further comprising administering a cancer immunotherapeutic agent.

4. The method of claim 1, wherein the cancer is brain cancer.

5. The method of claim 1, wherein the cancer is lung cancer.

6. The method of claim 1, wherein the cancer is ovary cancer.

7. The method of claim 1, wherein the cancer is breast cancer.

8. The method of claim 1, wherein the cancer is prostate cancer.

9. The method of claim 1, wherein the cancer is colon cancer.

10. The method of claim 1, wherein the cancer is leukemia.

11. The method of claim 1, wherein the cancer is carcinoma.

12. The method of claim 1, wherein the cancer is sarcoma.

13. The method of claim 1, wherein at least one nucleotide has a phosphate backbone modification.

14. The method of claim 13, wherein the phosphate backbone modification is a phosphorothioate or phosphorodithioate modification.

15. The method of claim 14, wherein the nucleic acid backbone includes the phosphate backbone modification on the 5' inter-nucleotide linkages.

16. The method of claim 14, wherein the nucleic acid backbone includes the phosphate backbone modification on the 3' inter-nucleotide linkages.

17. The method of claim 1, wherein the oligonucleotide has 8 to 100 nucleotides.

18. The method of claim 1, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

19. The method of claim 1, wherein X_{1X2} are GpA and X_{3X4} are TpT.

20. The method of claim 1, wherein X_{1X2} are both purines and X_{3X4} are both pyrimidines.

21. The method of claim 1, wherein X_{1X2} are GpA and X_{3X4} are both pyrimidines.

22. The method of claim 1, wherein the oligonucleotide is 8 to 40 nucleotides in length.

23. The method of claim 1, wherein the oligonucleotide is isolated.

24. The method of claim 1, wherein the oligonucleotide is a synthetic oligonucleotide.

25. A method for enhancing recovery of bone marrow using an **immunostimulatory** nucleic acid as compared to the absence of the **immunostimulatory** nucleic acid in a subject undergoing or having undergone cancer therapy, comprising: administering to a subject undergoing or having undergone cancer therapy which damages the bone marrow an effective amount for enhancing the recovery of bone marrow of an **immunostimulatory** nucleic acid, comprising: $5'X_{1X2CGX3X43}'$ wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides.

26. The method of claim 25, wherein at least one nucleotide has a phosphate backbone modification.

27. The method of claim 26, wherein the phosphate backbone modification is a phosphorothioate or phosphorodithioate modification.

28. The method of claim 25, wherein the oligonucleotide has 8 to 100 nucleotides.

29. The method of claim 25, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

30. In a method for stimulating an immune response in a subject having a cancer, the method of the type involving antigen dependent cellular cytotoxicity (ADCC), the improvement comprising: administering to the subject an **immunostimulatory** nucleic acid, comprising: $5'X_{1X2CGX3X43}'$ wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides.

31. The method of claim 30, wherein at least one nucleotide has a phosphate backbone modification.

32. The method of claim 30, wherein the oligonucleotide has 8 to 100 nucleotides.

33. The method of claim 32, wherein the phosphate backbone modification is a phosphorothioate or phosphorodithioate modification.

34. The method of claim 32, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

35. The method of claim 30, wherein 5' $X_{1X2CGX3X4}$ 3' is not palindromic.

36. A method for treating or preventing cancer, comprising: administering to a subject having a cancer an effective amount for treating or preventing cancer of an **immunostimulatory** nucleic acid, comprising: 5'$X_{1X2CGX3X43}$' wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides, and wherein the sequence is not palindromic.

37. The method of claim 36, further comprising administering a chemotherapeutic agent.

38. The method of claim 36, further comprising administering a cancer immunotherapeutic agent.

39. The method of claim 36, wherein the cancer is brain cancer.

40. The method of claim 36, wherein the cancer is lung cancer.

41. The method of claim 36, wherein the cancer is ovarian cancer.

42. The method of claim 36, wherein the cancer is breast cancer.

43. The method of claim 36, wherein the cancer is prostate cancer.

44. The method of claim 36, wherein the cancer is colon cancer.

45. The method of claim 36, wherein the cancer is leukemia.

46. The method of claim 36, wherein the cancer is carcinoma.

47. The method of claim 36, wherein the cancer is sarcoma.

48. The method of claim 36, wherein at least one nucleotide has a phosphate backbone modification.

49. The method of claim 48, wherein the phosphate backbone modification is a phosphorothioate or phosphorodithioate modification.

50. The method of claim 49, wherein the nucleic acid backbone includes the phosphate backbone modification on the 5' inter-nucleotide linkages.

51. The method of claim 49, wherein the nucleic acid backbone includes the phosphate backbone modification on the 3' inter-nucleotide linkages.

52. The method of claim 36, wherein the oligonucleotide has 8 to 100 nucleotides.

53. The method of claim 36, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

54. The method of claim 36, wherein X_{1X2} are GpA and X_{3X4} are TpT.

55. The method of claim 36, wherein X_{1X2} are both purines and X_{3X4} are both pyrimidines.

56. The method of claim 36, wherein X_{1X2} are GpA and X_{3X4} are both pyrimidines.

57. The method of claim 36, wherein the oligonucleotide is 8 to 40 nucleotides in length.

TI Method of treating cancer using **immunostimulatory** oligonucleotides

AI US 1999-337619 19990621 (9)

AB Nucleic acid sequences containing unmethylated **CpG** dinucleotides that modulate an immune response including stimulating a Th1 pattern of immune activation, cytokine production, NK lytic activity, and. . .

SUMM . . . present invention relates generally to oligonucleotides and more specifically to oligonucleotides which have a sequence including at least one unmethylated **CpG** dinucleotide which are **immunostimulatory**.

SUMM . . . CRE, the consensus form of which is the unmethylated sequence TGACGTC (SEQ. ID. No. 103) (binding is abolished if the **CpG** is methylated) (Iguchi-Ariga, S. M. M., and W. Schaffner: "**CpG** methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA (SEQ. ID. No.104) abolishes specific factor binding as well as transcriptional activation." Genese. . .

SUMM The present invention is based on the finding that certain nucleic acids containing unmethylated cytosine-guanine (**CpG**) dinucleotides activate lymphocytes in a subject and redirect a subject's immune response from a Th2 to a Th1 (e.g., by. . . to produce Th1 cytokines, including IL-12, IFN-γ and GM-CSF). Based on this finding, the invention features, in one aspect, novel **immunostimulatory** nucleic acid compositions.

SUMM In one embodiment, the invention provides an isolated **immunostimulatory** nucleic acid sequence containing a **CpG** motif represented by the formula:

SUMM In another embodiment, the invention provides an isolated **immunostimulatory** nucleic acid sequence contains a **CpG** motif represented by the formula:

SUMM In another embodiment, autoimmune disorders are treated by inhibiting a subject's response to **CpG** mediated leukocyte activation. The invention provides administration of inhibitors of endosomal acidification such as bafilomycin a, chloroquine, and monensin to. . .

DRWD FIG. 1B. Control phosphodiester oligodeoxynucleotide (ODN) 5' ATGGAAGGTCCAGTGTTCTC 3' (SEQ ID NO:1 14) (.box-solid.) and two phosphodiester **CpG** ODN 5' ATCGACCTACGTGCGTTCTC 3' (SEQ ID NO:2) (.diamond-solid.) and 5' TCCATAACGTTCCTGATGCT 3' (SEQ ID NO:3) (.circle-solid.).

DRWD FIG. 1C. Control phosphorothioate ODN 5' GCTAGATGTTAGCGT 3' (SEQ ID NO:4) (.box-solid.) and two phosphorothioate **CpG** ODN 5' GAGAACGTCGACCTTCGAT 3' (SEQ ID NO: 5) (.box-solid.) and 5' GCATGACGTTGAGCT 3' (SEQ ID NO:6) (.circle-solid.). Data present the. . .

DRWD FIG. 2 is a graph plotting IL-6 production induced by **CpG** DNA in vivo as determined 1-6 hrs after injection. Data represent the mean for duplicate analyses of sera from two mice. BALB/c mice (two mice/group) were inject iv. with 100 μl of PBS (.quadrature.) of 200 μg of **CpG** phosphorothioate ODN 5' TCCATGACGTTCCTGATGCT 3' (SEQ ID NO:7) (.box-solid.) or non-**CpG** phosphorothioate 5' TCCATGAGCTTCCTGAGTCT 3' (SEQ ID NO: 8) (.diamond-solid.).

DRWD . . . periods after in vivo stimulation of BALB/c mice (two mice/group) injected iv with 100 μl of PBS, 200 μl of **CpG** phosphorothioate ODN 5' TCCATGACGTTCCTGATGCT 3' (SEQ ID NO: 7) or non-**CpG** phosphorothioate ODN 5' TCCATGAGCTTCCTGAGTCT 3' (SEQ ID NO: 8).

DRWD FIG. 4A is a graph plotting dose-dependent inhibition of **CpG**-induced IgM production by anti-IL-6. Splenic B-cells from DBA/2 mice were stimulated with **CpG** ODN 5' TCCAAGACGTTCCTGATGCT 3' (SEQ ID NO:9) in the presence of the indicated concentrations of neutralizing anti-IL-6 (.diamond-solid.) or isotype control Ab (.circle-solid.) and IgM levels in culture supernatants determined by ELISA. In the absence of **CpG** ODN, the anti-IL-6 Ab had no effect on IgM secretion (.box-solid.).

DRWD FIG. 4B is a graph plotting the stimulation index of **CpG**-induced splenic B cells cultured with anti-IL-6 and **CpG** S--ODN 5' TCCATGACGTTCCTGATGCT 3' (SEQ ID NO:7) (.diamond-solid.) or anti-IL-6 antibody only (.box-solid.). Data present the mean±standard deviation of triplicates.

DRWD . . . cells transfected with a promoter-less CAT construct (pCAT), positive control plasmid (RSV), or IL-6 promoter-CAT construct alone or cultured with **CpG** 5' TCCATGACGTTCCTGATGCT 3' (SEQ ID NO: 7) or non-**CpG** 5' TCCATGAGCTTCCTGAGTCT 3' (SEQ ID NO: 8) phosphorothioate ODN at the indicated concentrations. Data present the mean of triplicates.

DRWD FIG. 6 is a schematic overview of the immune effects of the **immunostimulatory** unmethylated **CpG** containing nucleic acids, which can directly activate both B cells and monocytic cells (including macrophages and dendritic cells) as shown. The **immunostimulatory** oligonucleotides do not directly activate purified NK cells, but render them competent to respond to IL-12 with a marked increase in their

IFN-γ secretion by NK cells, the **immunostimulatory** nucleic acids promote a Th1 type immune response. No direct activation of proliferation of cytokine secretion by highly purified T cells has been found. However, the induction of Th1 cytokine secretion by the **immunostimulatory** oligonucleotides promotes the development of a cytotoxic lymphocyte response.

DRWD FIG. 7 is an autoradiograph showing NFκB mRNA induction in monocytes treated with E. coli (EC) DNA (containing unmethylated **CpG** motifs), control (CT) DNA (containing no unmethylated **CpG** motifs) and lipopolysaccharide (LPS) at various measured times, 15 and 30 minutes after contact.

DRWD . . . the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the **CpG** oligo (**TCCATGACGTTCCTGACGTT** SEQ ID NO: 10) also showed an increase in the level of reactive oxygen species such that more than 50%. . .

DRWD . . . have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with **CpG** DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and. . .

DRWD . . . egg antigen "SEA" (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given **CpG** oligo (SEQ ID NO: 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation. . .

DRWD . . . and subsequently inhale SEA (open circle), many eosinophils are present in the lungs. However, when the mice are initially given **CpG** oligo (SEQ ID NO: 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation. . .

DRWD . . . inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a **CpG** oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes. . .

DRWD . . . or SEQ ID NO. 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can actually redirect the cytokine response of the lung to production of IL-12, indicating a Th1 type of immune. . .

DRWD . . . or SEQ ID NO: 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can also redirect the cytokine response of the lung to production of IFN-g, indicating a Th1 type of immune. . .

DETD An "**immunostimulatory** nucleic acid molecule" refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (i.e., "**CpG** DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates (e.g., has a motogenic effect on, or induces or increases cytokine expression by) a vertebrate lymphocyte. An **immunostimulatory** nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have. . .

DETD In one preferred embodiment, the invention provides an isolated **immunostimulatory** nucleic acid sequence containing a **CpG** motif represented by the formula:

DETD In another embodiment the invention provides an isolated **immunostimulatory** nucleic acid sequence contains a **CpG** motif represented by the formula:

DETD Preferably, the **immunostimulatory** nucleic acid sequences of the invention include X_1X_2 selected from the group consisting of GpT, GpG, GpA and ApA and. . . selected from the group consisting of TpT, CpT and GpT (see for example, Table 5). For facilitating uptake into cells, **CpG** containing **immunostimulatory** nucleic acid molecules are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size (even many kb long) are **immunostimulatory** if sufficient **immunostimulatory** motifs are present, since such larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not. . . or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus mitogenic **CpG** motif is not a palindrome. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, where the oligonucleotide incorporates a phosphate backbone. . .

DETD Preferably the **immunostimulatory** **CpG** DNA is in the range of between 8 to 30 bases in size when it is an oligonucleotide. Alternatively, **CpG** dinucleotides can be produced on a large scale in plasmids, which after being administered to a subject are degraded into oligonucleotides. Preferred **immunostimulatory** nucleic acid molecules (e.g., for use in increasing the effectiveness of a vaccine or to treat an immune system deficiency. . .

DETD . . . useful as an adjuvant for use during antibody production in a mammal. Specific, but non-limiting examples of such sequences include: **TCCATGACGTTCCTGACGTT** (SEQ ID NO: 10), GTCGTT (SEQ. ID. NO: 57), GTCGCT (SEQ. ID. NO.58), TGTCGCT (SEQ. ID. NO: 101) and TGTCGTT. . .

symptoms of an asthmatic disorder by redirecting a subject's immune response from Th2 to Th 1. An exemplary sequence includes **TCCATGACGTTCCTGACGTT** (SEQ ID NO: 10).

DETD The stimulation index of a particular **immunostimulatory CpG** DNA can be tested in various immune cell assays. Preferably, the stimulation index of the **immunostimulatory CpG** DNA with regard to B-cell proliferation is at least about 5, preferably at least about 10, more preferably at least. . . treat an immune system deficiency by stimulating a cell-mediated (local) immune response in a subject, it is important that the **immunostimulatory CpG** DNA be capable of effectively inducing cytokine secretion by monocytic cells and/or Natural Killer (NK) cell lytic activity.

DETD Preferred **immunostimulatory CpG** nucleic acids should effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml. . . IL-6, 200 pg/ml IL-12, depending on the therapeutic indication, as determined by the assays described in Example 12. Other preferred **immunostimulatory CpG** DNAs should effect at least about 10%, more preferably at least about 15% and most preferably at least about 20%. . .

DETD . . . in vivo degradation (e.g., via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated **CpG** containing nucleic acid molecules that are tens to hundreds of kbs long are relatively resistant to in vivo degradation. For shorter **immunostimulatory** nucleic acid molecules, secondary structure can stabilize and increase their effect. For example, if the 3' end of a nucleic. . .

DETD . . . acid molecules (including phosphorodithioate-modified) can increase the extent of immune stimulation of the nucleic acid molecule, which contains an unmethylated **CpG** dinucleotide as shown herein. International Patent Application Publication Number WO 95/26204 entitled "Immune Stimulation By Phosphorothioate Oligonucleotide Analogs" also reports on the non-sequence specific **immunostimulatory** effect of phosphorothioate modified oligonucleotides. As reported herein, unmethylated **CpG** containing nucleic acid molecules having a phosphorothioate backbone have been found to preferentially activate B-cell activity, while unmethylated **CpG** containing nucleic acid molecules having a phosphodiester backbone have been found to preferentially activate monocytic (macrophages, dendritic cells and monocytes) and NK cells. Phosphorothioate **CpG** oligonucleotides with preferred human motifs are also strong activators of monocytic and NK cells.

DETD Certain Unmethylated **CpG** Containing Nucleic Acids Have B Cell Stimulatory Activity as Shown in vitro and in vivo

DETD . . . the other nonstimulatory control ODN. In comparing these sequences, it was discovered that all of the four stimulatory ODN contained **CpG** dinucleotides that were in a different sequence context from the nonstimulatory control.

DETD To determine whether the **CpG** motif present in the stimulatory ODN was responsible for the observed stimulation, over 300 ODN ranging in length from 5 to 42 bases that contained methylated, unmethylated, or no **CpG** dinucleotides in various sequence contexts were synthesized. These ODNs, including the two original "controls" (ODN 1 and 2) and two. . . then examined for in vitro effects on spleen cells (representative sequences are listed in Table 1). Several ODN that contained **CpG** dinucleotides induced B cell activation and IgM secretion; the magnitude of this stimulation typically could be increased by adding more **CpG** dinucleotides (Table 1; compare ODN 2 to 2a or 3D to 3Da and 3Db). Stimulation did not appear to result. . .

DETD Mitogenic ODN sequences uniformly became nonstimulatory if the **CpG** dinucleotide was mutated (Table 1; compare ODN 1 to 1a; 3D to 3Dc; 3M to 3Ma; and 4 to 4a) or if the cytosine of the **CpG** dinucleotide was replaced by 5-methylcytosine (Table 1; ODN 1b, 2b, 3Dd, and 3Mb). Partial methylation of **CpG** motifs caused a partial loss of stimulatory effect (compare 2a to 2c, Table 1). In contrast, methylation of other cytosines did not reduce ODN activity (ODN 1c, 2d, 3De and 3Mc). These data confirmed that **CpG** motif is the essential element present in ODN that activate B cells.

DETD In the course of these studies, it became clear that the bases flanking the **CpG** dinucleotide played an important role in determining the murine B cell activation induced by an ODN. The optimal stimulatory motif was determined to consist of a **CpG** flanked by two 5' purines (preferably a GpA dinucleotide) and two 3' pyrimidines (preferably a TpT or TpC dinucleotide). Mutations of ODN to bring the **CpG** motif closer to this ideal improved stimulation (e.g., Table 1, compare ODN 2 to 2e; 3M to 3Md) while mutations. . . Table 1, compare ODN 3D to 3Df; 4 to 4b, 4c and 4d). On the other hand, mutations outside the **CpG** motif did not reduce stimulation (e.g., Table 1, compare ODN to 1d; 3D to 3Dg; 3M to 3Me). For activation. . .

DETD . . . 10 As. The effect of the G-rich ends is cis; addition of an ODN

with poly G ends but no **CpG** motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated **CpG** were found to be more **immunostimulatory**.

DETD . . . from at least 3 separate experiments, and are compared to wells cultured with no added ODN. ND = not done. **CpG** dinucleotides are underlined. Dots indicate identity; dashes indicate deletions. Z = 5 methyl cytosine.

DETD

TABLE 2

Identification of the optimal **CpG** motif for Murine IL-6 production and B cell activation.

ODN SEQUENCE (5'-3')	IL-6 (pg/ml)[a] CH12.LX	SPLENIC B CELL SI[b]	IgM (ng/ml)[c]
512. . .	3534 ± 217		
1708 (SEQ ID No:40	.....CA..TG.....	ND	59 ± 3 1.5 ± 0.1 466 ± 109

Dots indicate identity; **CpG** dinucleotides are underlined; ND = not done

[a]The experiment was done at least three times with similar results. The level. . . both CH12.LX and splenic B cells was ≤10 pg/ml. The IgM level of unstimulated culture was 547 ± 82 ng/ml. **CpG** dinucleotides are underlined and dots indicate identity. Cells were stimulated with 20 μM of various **CpG** O-ODN. Data present the mean ± SD of triplicates.

[b][3H] Uridine uptake was indicated as a fold increase (SI: stimulation. . .

DETD . . . the subsequent fall in stimulation when purified B cells with or without anti-IgM (at a submitogenic dose) were cultured with **CpG** ODN, proliferation was found to synergistically increase about 10-fold by the two mitogens in combination after 48 hours. The magnitude. . .

DETD Cell cycle analysis was used to determine the proportion of B cells activated by **CpG**-ODN. **CpG**-ODN induced cycling in more than 95% of B cells. Splenic B lymphocytes sorted by flow cytometry into CD23- (marginal zone). . . as were both resting and activated populations of B cells isolated by fractionation over Percoll gradients. These studies demonstrated that **CpG**-ODN induced essentially all B cells to enter the cell cycle.

DETD **Immunostimulatory** Nucleic Acid Molecules Block Murine B Cell Apoptosis

DETD . . . are rescued from this growth arrest by certain stimuli such as LPS and by the CD40 ligand. ODN containing the **CpG** motif were also found to protect WEHI-231 from anti-IgM induced growth arrest, indicating that accessory cell populations are not required for the effect. Subsequent work indicates that **CpG** ODN induce Bcl-x and myc expression, which may account for the protection from apoptosis. Also, **CpG** nucleic acids have been found to block apoptosis in human cells. This inhibition of apoptosis is important, since it should enhance and prolong immune activation by **CpG** DNA.

DETD Identification of the Optimal **CpG** Motif for Induction of Murine IL-6 and IgM Secretion and B Cell Proliferation

DETD To evaluate whether the optimal B cell stimulatory **CpG** motif was identical with the optimal **CpG** motif for IL-6 secretion, a panel of ODN in which the bases flanking the **CpG** dinucleotide were progressively substituted was studied. This ODN panel was analyzed for effects on B cell proliferation, Ig production, and. . . using both splenic B cells and CH12.LX cells. As shown in Table 2, the optimal stimulatory motif contains an unrmethylated **CpG** flanked by two 5' purines and two 3' pyrimidines. Generally a mutation of either 5' purines to C were especially. . . had less marked effects. Based on analyses of these and scores of other ODN, it was determined that the optimal **CpG** motif for induction of IL-6 secretion is TGACGTT (SEQ. ID. NO: 108), which is identical with the optimal mitogenic and IgM-inducing **CpG** motif (Table 2). This motif was more stimulatory than any of the palindrome containing sequences studied (1639, 1707 and 1708).

DETD Induction of Murine Cytokine Secretion by **CpG** Motifs in Bacterial DNA or Oligonucleotides

DETD As described in Example 9, the amount of IL-6 secreted by spleen cells after **CpG** DNA stimulation was measured by ELISA. T cell depleted spleen cell cultures rather than whole spleen cells were used for in vitro studies following preliminary studies showing that T cells contribute little or nothing to the IL-6 produced by **CpG** DNA-stimulated spleen cells. As shown in Table 3, IL-6 production was markedly increased in cells cultured with E. coli DNA. . . response to bacterial DNA. To analyze whether the IL-6 secretion induced by E. coli DNA was mediated by the unmethylated **CpG** dinucleotides in bacterial DNA, methylated E. coli DNA and a panel of synthetic ODN were examined. As shown in Table 3, **CpG** ODN significantly induced IL-6 secretion (ODN 5a, 5b, 5c) while **CpG** methylated E. coli DNA, or ODN

containing methylated **CpG** (ODN 5f) or no **CpG** (ODN 5d) did not. Changes at sites other than **CpG** dinucleotides (ODN 5b) or methylation of other cytosines (ODN 5g) did not reduce the effect of **CpG** ODN. Methylation of a single **CpG** in an ODN with three CpGs resulted in a partial reduction in the stimulation (compare ODN 5c to 5e; Table. . .

DETD
TABLE 3

Induction of Murine IL-6 secretion by **CpG** motifs
in bacterial DNA or oligonucleotides.

Treatment	IL-6 (pg/ml)
calf thymus DNA	≤10
calf thymus DNA + DNase	≤10
E. coli DNA	1169.5 ± 94.1
E. coli DNA + DNase	≤10
CpG methylated E. coli DNA	≤10
LPS	280.1 ± 17.1
Media (no DNA)	≤10

5a SEQ. ID. ATGGACTCTCCAGCGTTCTC 1096.4 ± 372.0
No:115

5b. . . or without enzyme treatment, or LPS (10 μg/ml) for 24 hr. Data represent the mean (pg/ml) ± SD of triplicates. **CpG** dinucleotides are underlined and dots indicate identity. Z indicates 5-methylcytosine.

DETD **CpG** Motifs can be Used as an Artificial Adjuvant

DETD . . . more acceptable side effects has led to the production of new synthetic adjuvants. The present invention provides the sequence 1826. **TCCATGACGTTCCTGACGTT** (SEQ ID NO: 10), which is an adjuvant including **CpG** containing nucleic acids. The sequence is a strong immune activating sequence and is a superb adjuvant, with efficacy comparable or. . .

DETD Titration of Induction of Murine IL-6 Secretion by **CpG** Motifs

DETD Bacterial DNA and **CpG** ODN induced IL-6 production in T cell depleted murine spleen cells in a dose-dependent manner, but vertebrate DNA and non-**CpG** ODN did not (FIG. 1). IL-6 production plateaued at approximately 50 μg/ml of bacterial DNA or 40 μM of **CpG** O--ODN. The maximum levels of IL-6 induced by bacterial DNA and **CpG** ODN were 1-1.5 ng/ml and 2-4 ng/ml respectively. These levels were significantly greater than those seen-after stimulation by LPS (0.35 ng/ml) (FIG. 1A). To evaluate whether **CpG** ODN with a nuclease-resistant DNA backbone would also induce IL-6 production, S--ODN were added to T cell depleted murine spleen cells. **CpG** S--ODN also induced IL-6 production in a dose-dependent manner to approximately the same level as **CpG** O--ODN while non-**CpG** S--ODN failed to induce IL-6 (FIG. 1C). **CpG** S--ODN at a concentration of 0.05 μM could induce maximal IL-6 production in these cells. This result indicted that the nuclease-resistant DNA backbone modification retains the sequence specific ability of **CpG** DNA to induce IL-6 secretion and that **CpG** S--ODN are more than 80-fold more potent than **CpG** O--ODN in this assay system.

DETD Induction of Murine IL-6 Murine by **CpG** DNA in vivo

DETD To evaluate the ability of bacterial DNA and **CpG** S--ODN to induce Il-6 secretion in vivo, BALB/c mice were injected iv. with 100 μg of E. coli DNA, calf thymus DNA, or **CpG** or non-stimulatory S--ODN and bled 2 hr after stimulation. The level of IL-6 in the sera from the E. coli. . . 13 ng/ml while IL-6 was not detected in the sera from calf thymus DNA or PBS injected groups (Table 4). **CpG** S--ODN also induced IL-6 secretion in vivo. The IL-6 level in the sera from **CpG** S--ODN injected groups was approximately 20 ng/ml. In contrast, IL-6 was not detected in the sera from non-stimulatory S--ODN stimulated. . .

DETD
TABLE 4

Secretion of Murine IL-6 induced by **CpG** DNA
stimulation in vivo.

Stimulant	IL-6 (pg/ml)
PBS	<50
E. coliDNA	13858 ± 3143
Calf Thymus DNA	<50
CpG S-ODN	20715 ± 606
non-**CpG** S-ODN	<50

Mice (2 mice/group) were i.v. injected with 100 μl of PBS, 200 μl of E. coli DNA or calf thymus DNA, or 500 μg of **CpG** S-ODN or non-**CpG** control S-ODN. Mice were bled 2 hr after injection and 1:10 dilution of each serum was analyzed by IL-6 ELISA. Sensitivity limit of IL-6 ELISA

was 5 pg/ml. Sequences of the CpG S-ODN is 5'GCATGACGTTGAGCT3'(SEQ. ID. No:6) and of the non-stimulatory S-ODN is 5'GCTAGATGTTAGCGT3'(SEQ. ID. No:4). Note that although there is a CpG in sequence 48, # it is too close to the 3' end to effect stimulation, as explained herein. Data. . .

DETD Kinetics of Murine IL-6 Secretion After Stimulation by CpG Motifs in vivo

DETD To evaluate the kinetics of induction of IL-6 secretion by CpG DNA n vivo, BALB/c mice were injected iv. with CpG or control non-CpG S--ODN. Serum IL-6 levels were significantly increased within 1 hr and peaked at 2 hr to a level of approximately 9 ng/ml in the CpG S--ODN injected group (FIG. 2). Il-6 protein in sera rapidly decreased after 4 hr and returned to basal level by 12 hr after stimulation. In contrast to CpG DNA stimulated groups, no significant increase of IL-6 was observed in the sera from the non-stimulatory S--ODN or PBS injected. .

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DETD Tissue Distribution and Kinetics of IL-6, mRNA Expression Induced by CpG Motifs in vivo

DETD As shown in FIG. 2, the level of serum IL-6 increased rapidly after CpG DNA stimulation. To investigate the possible tissue origin of this serum IL-6, and the kinetics of IL-6 gene expression in vivo after CpG DNA stimulation, BALB/c mice were injected iv with CpG or non-CpG S--ODN and RNA was extracted from liver, spleen, thymus, and bone marrow at various time points after stimulation. As shown. . . FIG. 3A, the level of IL-6 mRNA in liver, spleen, and thymus was increased within 30 min. after injection of CpG S--ODN. The liver IL-6 mRNA peaked at 2 hr post-injection and rapidly decreased and reached basal level 8 hr after. . . hr post-injection and then gradually decreased (FIG. 3A). IL-6 mRNA was significantly increased in bone marrow within 1 hr after CpG S--ODN injection but then returned to basal level. In response to CpG S--ODN, liver, spleen and thymus showed more substantial increases in IL-6 mRNA expression than the bone marrow.

DETD Patterns of Murine Cytokine Expression Induced by CpG DNA

DETD . . . within 30 minutes and the level of IL-6 increased strikingly within 2 hours in the serum of mice injected with CpG ODN. Increased expression of IL-12 and interferon gamma (IFN-γ) mRNA by spleen cells was also detected within the first two. . .

DETD . . . 0 70

SEQ ID NO:39

1708 . . . CA_TG . . . 270 10 17 ND 10

SEQ ID NO:40

dots indicate identity; CpG dinucleotides are underlined

1measured by ELISA using Quantikine kits from R&D Systems (pg/ml) Cells were cultured in 10% autologous serum. . .

DETD CpG DNA Induces Cytokine Secretion by Human PBMC, Specifically Monocytes

DETD . . . panels of ODN used for studying mouse cytokine expression were used to determine whether human cells also are induced by CpG motifs to express cytokine (or proliferate), and to identify the CpG motif(s) responsible. Oligonucleotide 1619 (GTCGTT; SEQ. ID. NO: 57) was the best inducer of TNF-α and IFN-γ secretion, and was. . . little induction of other cytokines. Thus, it appears that human lymphocytes, like murine lymphocytes, secrete cytokines differentially in response to CpG dinucleotides, depending on the surrounding bases. Moreover, the motifs that stimulate murine cells best differ from those that are most effective with human cells. Certain CpG oligodeoxynucleotides are poor at activating human cells (oligodeoxynucleotides 1707, 1708, which contain the palindrome forming sequences GACGTC (SEQ. ID. NO:. . .

DETD . . . simply reflect a nonspecific death of all cell types. Cytokine secretion in response to E. coli (EC) DNA requires unmethylated CpG motifs, since it is abolished by methylation of the EC DNA (next to the bottom row, Table 6). LPS contamination. . .

DETD

TABLE 6

CpG DNA induces cytokine secretion by human PBMC

DNA	TNF-α (pg/ml)[1]	IL-6 (pg/ml)	IFN-γ (pg/ml)	RANTES (pg/ml)

EC DNA (50 μg/ml) 900 12,000. . . cytokine production under these conditions was from monocytes (or other L-LME-sensitive cells).

3EC DNA was methylated using 2U/μg DNA of CpG methylase (New England Biolabs) according to the manufacturer's directions, and methylation confirmed by digestion with Hpa-II and Msp-I. As a. . .

DETD . . . cytokine production in the PBMC treated with L-LME suggested that monocytes may be responsible for cytokine production in response to CpG DNA. To test this hypothesis more directly, the effects of CpG DNA on highly purified human monocytes and macrophages was tested. As hypothesized, CpG DNA directly activated production of the cytokines

IL-6, GM-CSF, and TNF-α by human macrophages, whereas non-**CpG** DNA did not (Table 7).

DETD

TABLE 7

CpG DNA induces cytokine expression in purified human macrophages
IL-6 (pg/ml) GM-CSF (pg/ml) TNF-α(pg/ml)

Cells alone 0 0 0
CT DNA (50 μg/ml). . .

DETD Biological Role of IL-6 in Inducing Murine IgM Production in Response to **CpG** Motifs

DETD The kinetic studies described above revealed that induction of IL-6 secretion, which occurs within 1 hr post **CpG** stimulation, precedes IgM secretion. Since the optimal **CpG** motif for ODN inducing secretion of IL-6 is the same as that for IgM (Table 2), whether the **CpG** motifs independently induce IgM and IL-6 production or whether the IgM production is dependent on prior IL-6 secretion was examined. The addition of neutralizing anti-IL-6 antibodies inhibited in vitro IgM production mediated by **CpG** ODN in a dose-dependent manner but a control antibody did not (FIG. 4A). In contrast, anti-IL-6 addition did not affect either the basal level or the **CpG**-induced B cell proliferation (FIG. 4B).

DETD Increased Transcriptional Activity of the IL-6 Promoter in Response to **CpG** DNA

DETD The increased level of IL-6 mRNA and protein after **CpG** DNA stimulation could result from transcriptional or post-transcriptional regulation. To determine if the transcriptional activity of the IL-6 promoter was unregulated in B cells cultured with **CpG** ODN, a murine B cell line, WEHI-231, which produces IL-6 in response to **CpG** DNA, was transfected with an IL-6 promoter-CAT construct (pIL-6/CAT) (Pottrats, S. T. et al, 17B-estradiol) inhibits expression of human interleukin-6-promoter-reporter constructs by a receptor-dependent mechanism. J. Clin. Invest. 93:944). CAT assays were performed after stimulation with various concentrations of **CpG** or non-**CpG** ODN. As shown in FIG. 5, **CpG** ODN induced increased CAT activity in dose-dependent manner while non-**CPG** ODN failed to induce CAT activity. This confirms that **CpG** induces the transcriptional activity of the IL-6 promoter.

DETD Dependence of B Cell Activation by **CpG** ODN on the Number of 5' and 3' Phosphorothioate Internucleotide Linkages

DETD . . . or DNA synthesis (by ^{3H} thymidine incorporation) in treated spleen cell cultures (Example 10). O--ODN (0/0 phosphorothioate modifications) bearing a **CpG** motif caused no spleen cell stimulation unless added to the cultures at concentrations of at least 10 μM (Example 10).. . .

DETD . . . result from the nuclease resistance of the former. To determine the role of ODN nuclease resistance in immune stimulation by **CpG** ODN, the stimulatory effects of chimeric ODN in which the 5' and 3' ends were rendered nuclease resistant with either. . .

DETD . . . while the S--ODN with the 3D sequence was less potent than the corresponding S--O--ODN (Example 10). In comparing the stimulatory **CpG** motifs of these two sequences, it was noted that the 3D sequence is a perfect match for the stimulatory motif in that the **CpG** is flanked by two 5' purines and two 3' pyrimidines. However, the bases immediately flanking the **CpG** in ODN 3D are not optimal; it has a 5' pyrimidine and a 3' purine. Based on further testing, it. . . for immune stimulation is more stringent for S--ODN than for S--O-- or O--ODN. S--ODN with poor matches to the optimal **CpG** motif cause little or no lymphocyte activation (e.g., Sequence 3D). However, S--ODN with good matches to the motif, most critically at the positions immediately flanking the **CpG**, are more potent than the corresponding S--O--ODN (e.g., Sequence 3M, Sequences 4 and 6), even though at higher concentrations (greater. . .

DETD The increased B cell stimulation seen with **CpG** ODN bearing S or S_2 substitutions could result from any or all of the following effects: nuclease resistance, increased cellular. . . localization. However, nuclease resistance cannot be the only explanation, since the MP--O--ODN were actually less stimulatory than the O--ODN with **CpG** motifs. Prior studies have shown that ODN uptake by lymphocytes is markedly affected by the backbone chemistry (Zhao, et al.. . .

DETD Unmethylated **CpG** Containing Oligos Have NK Cell Stimulatory Activity

DETD Experiments were conducted to determine whether **CpG** containing oligonucleotides stimulated the activity of natural killer (NK) cells in addition to B cells. As shown in Table 8, a marked induction of NK activity among spleen cells cultured with **CpG** ODN 1 and 3Dd was observed. In contrast, there was relatively on induction in effectors that had been treated with non-**CpG** control ODN.

DETD

TABLE 8

Induction Of NK Activity By **CpG** Oligodeoxynucleotides (ODN)
% YAC-1 % 2C11
Specific Lysis* Specific Lysis
Effector: Target Effector: Target
ODN 50:1 100:1 50:1 100:1

None -1.1 -1.4 15.3 16.6
1 16.1 24.5 38.7 47.2
3Dd 17.1 27.0 37.0 40.0
non-**CpG** ODN -1.6 -1.7 14.8 15.4

DETD Induction of NK Activity by DNA Containing **CpG** Motifs, but Not by Non-**CpG** DNA

DETD . . . depleted of B cells and human PBMC, but vertebrate DNA may be a consequence of its increased level of unmethylated **CpG** dinucleotides, the activating properties of more than 50 synthetic ODN containing unmethylated, methylated, or no **CpG** dinucleotides was tested. The results, summarized in Table 9, demonstrate that synthetic ODN can stimulate significant NK activity, as long as they contain at least one unmethylated **CpG** dinucleotide. No difference was observed in the stimulatory effects of ODN in which the **CpG** was within a palindrome (such as ODN 1585, which contains the palindrome AACGTT; SEQ. ID. NO: 105) from those ODN. . . palindromes (such as 1613 ro 1619), with the caveat that optimal stimulation was generally seem with ODN in which the **CpG** was flanked by two 5' purines or a 5' GpT dinucleotide and two 3' pyrimidines. Kinetic experiments demonstrated that NK. . . of the ODN. The data indicates that the murine NK response is dependent on the prior activation of monocytes by **CpG** DNA, leading to the production of IL-12, TNF-α, and IFN-α/b (Example 11).

DETD
TABLE 9

Induction of NK Activity by DNA Containing **CpG** Motifs but not by Non-**CpG** DNA
LU/10^6
DNA or Cytokine Added Mouse Cells Human Cells

Expt. 1 None 0.00 0.00
IL-2 16.68 15.82
E. Coli. DNA. . . -------Z------ (SEQ ID No. 117) 0.02 ND
1619 TCCATGTCGTTCCTGATGCT (SEQ ID No. 38) 3.35
1765 --------Z---------- (SEQ ID No. 44) 0.11

CpG dinucleotides in ODN sequences are indicated by underlying; Z indicates methylcytosine. Lower case letters indicate nuclease resistant phosphorothioate modified internucleotide. . .

DETD From all of these studies, a more complete understanding of the immune effects of **CpG** DNA has been developed, which is summarized in FIG. 6.

DETD Immune activation by **CpG** motifs may depend on bases flanking the **CpG**, and the number of spacing of the CpGs present within an ODN. Although a single **CpG** in an ideal base context can be a very strong and useful immune activator, superior effects can be seen with ODN containing several CpGs with the appropriate spacing and flanking bases. For activation of murine B cells, the optimal **CpG** motif is TGACGTT (SEQ. ID. NO: 108); residues 10-17 of Seq. ID. No 70.

DETD . . . ODN sequences for stimulation of human cells by examining the effects of changing the number, spacing, and flanking bases of **CpG** dinucleotides.

DETD Identification of Phosphorothioate ODN with Optimal **CpG** Motifs for Activation of Human NK Cells

DETD . . . cellular uptake (Krieg et al., Antisense Res. Dev. 6:133, 1996.) and improved B cell stimulation if they also have a **CpG** motif. Since NK activation correlates strongly with in vivo adjuvant effects, the identification of phosphorothioate ODN that will activate human. .

DETD The effects of different phosphorothioate ODNs--containing **CpG** dinucleotides in various base contexts--on human NK activation (Table 10) were examined. ODN 1840, which contained 2 copies of the. . . 10). To further identify additional ODNs optimal for NK activation, approximately one hundred ODN containing different numbers and spacing of **CpG** motifs, were tested with ODN1982 serving as a control. The result are shown in Table 1.

DETD . . . ODNs began with a TC or TG at the 5' end, however, this requirement was not mandatory. ODNs with internal **CpG** motifs (e.g., ODN 1840) are generally less potent stimulators than those in which a GTCGCT (SEQ. ID. NO: 58) motif. . . in which only one of the motifs had the additional of the spacing two Ts. The minimal acceptable spacing between **CpG** motifs is one nucleotide as long as the ODN has two pyrimidines (preferably T) at the 3' end (e.g., ODN. . . T also created a reasonably strong inducer of NK activity (e.g., ODN 2016). The

choice of thymine (T) separating consecutive **CpG** dinucleotides is not absolute, since ODN 2002 induced appreciable NK activation despite the fact that adenine (A) separated its CpGs (i.e., CGACGTT; SEQ. ID. NO: 113). It should also be noted that ODNs containing no **CpG** (e.g., ODN 1982), runs of CpGs, or CpGs in bad sequence contents (e.g., ODN 2010) had no stimulatory effect on. . .

DETD Table 11. Induction of NK LU by Phosphorothioate **CpG** ODN with Good Motifs

DETD

TABLE 11

Induction of NK LU by Phosphorothioate **CpG** ODN with Good Motifs

ODN[1] expt. 1 expt. 2 expt. 3

cells

alone sequence (5'-3') SEQ ID NO: 0.00 1.26 0.46

1840 TCCATGTCGTTCCTGTCGTT. . .

[2]This is the methylated version of ODN 1840; Z = 5-methyl cytosine LU is lytic units; ND = not done; **CpG** dinucleotides are underlined for clarity.

DETD Identification of Phosphorothioate ODN with Optimal **CpG** Motifs for Activation of Human B Cell Proliferation

DETD The ability of a **CpG** ODN to induce B cell proliferation is a good measure of its adjuvant potential. Indeed, ODN with strong adjuvant effects generally also induce B cell proliferation. To determine whether the optimal **CpG** ODN for inducing B cell proliferation are the same as those for inducing NK cell activity, similar panels of ODN. . .

DETD

TABLE 12

Induction of human B cell proliferation by Phosphorothioate **CpG** ODN

Stimulation Index[1]

ODN sequence (5'-3') SEQ ID NO: expt. 1 expt. 2 expt. 3 expt 4 expt. 5 expt. 6

1840. . .

DETD The ability of a **CpG** ODN to induce IL-12 secretion is a good measure of its adjuvant potential, especially in terms of its ability to. . . OIL-12 secretion from human PBMC in vitro (Table 13) was examined. These experiments showed that in some human PBMC, most **CpG** ODN could induce IL-12 secretion (e.g., expt. 1). However, other donors responded to just a few **CpG** ODN (E.g., expt. 2). ODN 2006 was a consistent inducer of IL12 secretion from most subjects (Table 13).

DETD

TABLE 13

Induction of human IL-12 secretion by Phosphorothioate **CpG** ODN

IL-12 (pg/ml)

ODN[1] expt. 1 expt. 2

cells alone sequence (5'-3') SEQ ID NO: 0 0

1962 TCCTGTCGTTCCTTGTCGTT 52 19 0

1965 TCCTGTCGTTTTTTGTCGTT. . .

DETD As shown in FIG. 6, **CpG** DNA can directly activate highly purified B cells and monocytic cells. There are many similarities in the mechanism through which **CpG** DNA activates these cell types. For example, both require NFκB activation as explained further below.

DETD In further studies of different immune effects of **CpG** DNA, it was found that there is more than one type of **CpG** motif. Specifically, olio 1668, with the best mouse B cell motif, is a strong inducer of both B cell and. . .

DETD

TABLE 14

Different **CpG** motifs stimulate optimal murine B cell and NK activation

ODN Sequence B cell activation NK activation

1668 TCCATGACGTTCCTGATGCT (SEQ.ID.NO 7) 42,849 2.52

1758 TCTCCCAGCGTGCGCCAT (SEQ.ID.NO.45) 1,747 6.66

NONE 367 0.00

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance. Measured by H thymidine incorporation. . .

DETD Teleological Basis of **Immunostimulatory**, Nucleic Acids

DETD Vertebrate DNA is highly methylated and **CpG** dinucleotides are under represented. However, the stimulatory **CpG** motif is common in microbial genomic DNA, but quite rare in vertebrate DNA. In addition, bacterial DNA has been reported. . . P. et al., J. Immunol. 147:1759 (1991)).

Experiments further described in Example 3, in which methylation of bacterial DNA with **CpG** methylase was found to abolish mitogenicity, demonstrates that the difference in **CpG** status is the cause of B cell stimulation by bacterial DNA. This data supports the following conclusion: that unmethylated **CpG** dinucleotides present within bacterial DNA are responsible for the stimulatory effects of bacterial DNA.

DETD Teleologically, it appears likely that lymphocyte activation by the **CpG** motif represents an immune defense mechanism that can thereby distinguish bacterial from host DNA. Host DNA, which would commonly be. . . regions and areas of inflammation due to apoptosis (cell death), would generally induce little or mo lymphocyte activation due to **CpG** suppression and methylation. However, the presence of bacterial DNA containing unmethylated **CpG** motifs can cause lymphocyte activation precisely in infected anatomic regions, where it is beneficial. This novel activation pathway provides a rapid alternative to T cell dependent antigen specific B cell activation. Since the **CpG** pathway synergizes with B cell activation through the antigen receptor, B cells bearing antigen receptor specific for bacterial antigens would. . .

DETD . . . 35:647 (1992)), is likely triggered at least in part by activation of DNA-specific B cells through stimulatory signals provided by **CpG** motifs, as well as by binding of bacterial DNA to antigen receptors.

DETD . . . products released from dying bacteria that reach concentrations sufficient to directly activate many lymphocytes. Further evidence of the role of **CpG** DNA in the sepsis syndrome is described in Cowdery, J., et. al., (1996) the Journal of Immunology 156:4570-4575.

DETD Unlike antigens that trigger B cells through their surface Ig receptor, **CpG**-ODN did not induce any detectable Ca^{2+} flux, changes in protein tyrosine phosphorylation, or IP 3 generation. Flow cytometry with FITC-conjugated ODN with or without a **CpG** motif was performed as described in Zhao, Q et al., (Antisense Research and Development 3:53-66 (1993)), and showed equivalent membrane binding, cellular uptake, efflux, and intracellular localization. This suggests that there may not be cell membrane proteins specific for **CpG** ODN. Rather than acting through the cell membrane, that data suggests that unmethylated **CpG** containing oligonucleotides require cell uptake for activity: ODN covalently linked to a solid Teflon support were nonstimulatory, as were biotinylated ODN immobilized on either avidin beads or avidin coated petri dishes. **CpG** ODN conjugated to either FITC or biotin retained full mitogenic properties, indicated no stearic hindrance.

DETD Recent data indicate the involvement of the transcription factor NFkB as a direct or indirect mediator of the **CpG** effect. For example, within 15 minutes of treating B cells or monocytes with **CpG** DNA, the level of NFkB binding activity is increased (FIG. 7). However, it is not increased by DNA that does not contain **CpG** motifs. In addition, it was found that two different inhibitors of NFkB activation, PDTC and gliotoxin, completely block the lymphocyte stimulation by **CpG** DNA as measured by B cell proliferation or monocytic cell cytokine secretion, suggesting that NFkB activation is required for both. . .

DETD . . . various protein kinases, or through the generation of reactive oxygen species. No evidence for protein kinase activation induced immediately after **CpG** DNA treatment of B cells or monocytic cells have been found, and inhibitors of protein kinase A, protein kinase C, and protein tyrosine kinases had no effects on the **CpG** induced activation. k However, **CpG** DNA causes a rapid induction of the production of reactive oxygen species in both B cells and monocytic cells, as. . . reactive oxygen species completely block the induction of NFkB and the later induction of cell proliferation and cytokine secretion by **CpG** DNA.

DETD Work backwards, the next question was how **CpG** DNA leads to the generation of reactive oxygen species so quickly. Previous studies by the inventors demonstrated that oligonucleotides and. . . rapidly become acidified inside the cell. To determine whether this acidification step may be important in the mechanism through which **CpG** DNA activates reactive oxygen species, the acidification step was blocked with specific inhibitors of endosome acidification including chloroquine, monensin, and. . . the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the **CpG** oligo also showed an increase in the level of reactive oxygen species such that more than 50% of the cells became positive (Panel D). However, cells treated with an oligonucleotide with the identical sequence except that the **CpG** was switched did not show this significant increase in the level of reactive oxygen species (Panel E).

DETD . . . have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with **CpG** DNA (Panel B) but does not reduce the level of reactive oxygen species in t he cells treated with PMA. . . This demonstrates that unlike the PMA plus ionomycin, the generation of reactive oxygen species following treatment of B cells with **CpG** DNA requires that the

DNA undergo an acidification step in the endosomes. This is a completely novel mechanism of leukocyte activation. Chloroquine, monensin, and bafilomycin also appear to block the activation of NFκB by **CpG** DNA as well as the subsequent proliferation and induction of cytokine secretion.

DETD Chronic Immune Activation by **CpG** DNA and Autoimmune Disorders

DETD B cell activation by **CpG** DNA synergizes with signals through the B cell receptor. This raises the possibility that DNA-specific B cells may be activated by the concurrent binding of bacterial DNA to their antigen receptor, and by the co-stimulatory **CpG**-mediated signals. In addition, **CpG** DNA induces B cells to become resistant to apoptosis, a mechanism thought to be important for preventing immune responses to self antigens, such as DNA. Indeed, exposure to bDNA can trigger anti-DNA Ab production. Given this potential ability of **CpG** DNA to promote autoimmunity, it is therefore noteworthy that patients with the autoimmune disease systemic lupus erythematosus have persistently elevated. . . circulating plasma DNA which is enriched in hypomethylated CpGs. These findings suggest a possible role for chronic immune activation by **CpG** DNA in lupus etiopathogenesis.

DETD . . . While the therapeutic mechanism of these drugs has been unclear, they are known to inhibit endosomal acidification. Leukocyte activation by **CpG** DNA is not medicated through binding to a cell surface receptor, but requires cell uptake, which occurs via adsorptive endocytosis into an acidified chloroquine-sensitive intracellular compartment. This suggested the hypothesis that leukocyte activation by **CpG** DNA may occur in association with acidified endosomes, and might even be pH dependent. To test this hypothesis specific inhibitors of DNA acidification were applied to determine whether B cells or monocytes could respond to **CpG** DNA if endosomal acidification was prevented.

DETD The earliest leukocyte activation event that was detected in response to **CpG** DNA is the production of reactive oxygen species (ROS), which is induced within five minutes in primary spleen cells and. . . cell lines. Inhibitors of endosomal acidification including chloroquine, bafilomycin A, and monensin, which have different mechanisms of action, blocked the **CpG**-induced generation of ROS, but had no effect on ROS generation mediated by PMA, or ligation of CD40 or IgM. These. . . diverse pathways. This ROS generation is generally independent of endosomal acidification, which is required only for the ROS response to **CpG** DNA. ROS generation in response to **CpG** is not inhibited by the NFκB inhibitor gliotoxin, confirming that it is not secondary to NFκB activation.

DETD To determine whether endosomal acidification of **CpG** DNA was also required for its other immune stimulatory effects were performed. Both LPS and **CpG** DNA induce similar rapid NFκB activation, increases in proto-oncogene mRNA levels, and cytokine secretion. Activation of NFκB by DNA depended on **CpG** motifs since it was not induced by bDNA treated with **CpG** methylase, nor by ODN in which bases were switched to disrupt the CpGs. Supershift experiments using specific antibodies indicated that the activated NFκB complexes included the p50 and p65 components. Not unexpectedly, NFκB activation in LPS- or **CpG**-treated cells was accompanied by the degradation of IκBα and IκBβ. However, inhibitors of endosomal acidification selectively blocked all of the **CpG**-induced but none of the LPS-induced cellular activation events. The very low concentration of chloroquine (<10 μM) that has been determined to inhibit **CpG**-mediated leukocyte activation is noteworthy since it is well below that required for antimalarial activity and other reported immune effects (e.g., 100-1000 μM). These experiments support the role of a pH-dependent signaling mechanism in mediating the stimulatory effects of **CpG** DNA.

DETD

TABLE 15

Specific blockade of **CpG**-induced TNF-αand IL-12 expression by inhibitors of endosomal acidification or NFκB activation

Inhibitors:							
	Bafilomycin	Chloroquine	Monensin	NAC	TPCK	Gliotoxin	Bisgliotoxin
. . .	IL-12	TNF-α IL-12	TNF-α	IL-12	TNF-α	TNF-α	
	TNF-α	TNF-α					
Medium	37	147 46	102 27	20	22	73	10 24 17 41
CpG	455	17,114 71	116 28	6	49	777	54 23 31 441
ODN							
LPS	901	22,485 1370	4051 1025	12418	491	4796. . .	

DETD . . . were cultured with or without the indicated inhibitors at the concentrations shown for 2 hr and then stimulated with the **CpG** oligodeoxynucleotide (ODN) 1826 (**TCCATGACGTTCCTGACGTT** SEQ ID NO:10) at 2 μM or LPS (10 μg/ml) for 4 hr (TNF-α) or 24 hr (IL-12) at which. . . Immunol., 157, 5394-5402 (1996); Krieg, A. M., J. Lab.

Clin. Med., 128, 128-133 (1996). Cells cultured with ODN that lacked **CpG** motifs did not induce cytokine secretion. Similar specific inhibition of **CpG** responses was seen with IL-6 assays, and in experiments using primary spleen cells or the B cell lines CH12.LX and. . .

DETD Excessive immune activation by **CpG** motifs may contribute to the pathogenesis of the autoimmune disease systemic lupus erythematosus, which is associated with elevated levels of circulating hypomethylated **CpG** DNA. Chloroquine and related antimalarial compounds are effective therapeutic agents for the treatment of systemic lupus erythematosus and some other. . . mechanism of action has been obscure. Our demonstration of the ability of extremely low concentrations of chloroquine to specifically inhibit **CpG**-mediated leukocyte activation suggests a possible new mechanism for its beneficial effect. It is noteworthy that lupus recurrences frequently are thought. . . bDNA present in infected tissues can be sufficient to induce a local inflammatory response. Together with the likely role of **CpG** DNA as a mediator of the sepsis syndrome and other diseases our studies suggest possible new therapeutic applications for the. . .

DETD **CpG**-induced ROS generation could be an incidental consequence of cell activation, or a signal that mediates this activation. The ROS scavenger N-acetyl-L-cysteine (NAC) blocks **CpG**-induced NFκB activation, cytokine production, and B cell proliferation, suggesting a casual role for ROS generation in these pathways. These data. . . gliotoxin (0.2 μg/ml). Cell aliquots were then cultured as above for 10 minutes in RPMI medium with or without a **CpG** ODN (1826) or non-**CpG** ODN (1911) at 1 μM or phorbol myristate acetate (PMA) plus ionomycin (iono). Cells were then stained with dihydrorhodamine-123 and. . . 5394-5402 (1996); Krieg, A. M, J. Lab. Clin. Med., 128, 128-133 (1996)). J1774 cells, a monocytic line, showed similar pH-dependent **CpG** induced ROS responses. In contrast, **CpG** DNA did not induce the generation of extracellular ROS, nor any detectable neutrophil ROS. The concentrations of chloroquine (and those used with the other inhibitors of endosomal acidification) prevented acidification of the internalized **CpG** DNA using fluorescein conjugated ODN as described by Tonkinson, et al., (Nucl. Acids Res. 22, 4268 (1994); A. M. Krieg,. . .

DETD While NFκB is known to be an important regulator of gene expression, it's role in the transcriptional response to **CpG** DNA was uncertain. To determine whether this NFκB activation was required for the **CpG** mediated induction of gene expression cells were activated with **CpG** DNA in the presence or absence of pyrrolidine dithiocarbamate (PDTC), an inhibitor of IκB phosphorylation. These inhibitors of NFκB activation completely blocked the **CpG**-induced expression of protooncogene and cytokine mRNA and protein, demonstrating the essential role of NFκB as a mediator of these events.. . . was cultured in the presence of calf thymus (CT), E. coli (EC), or methylated E. coli (mEC) DNA (methylated with **CpG** methylase as described) at 5 μg/ml or a **CpG** oligodeoxynucleotide (ODN 1826; Table 15) or a non-**CpG** ODN (ODN 1745; TCCATGAGCTTCCTGAGTCT, SEQ. ID. NO: 8) at 0.75 μM for 1 hr, following which the cells were lysed. . . was determined by supershifting with specific Ab to p65 and p50 (Santa Cruz Biotechnology, Santa Cruz, Calif.). Chloroquine inhibition of **CpG**-induced but not LPS-induced NFκB activation was established using J774 cells. The cells were precultured for 2 hr in the presence or absence of chloroquine (20 μg/ml) and then stimulated as above for 1 hr with either EC DNA, **CpG** ODN, non-**CpG** ODN or LPS (1 μg/ml). Similar chloroquine sensitive **CpG**-induced activation of NFκB was seen in a B cell line, WEHI-231 and primary spleen cells. These experiments were performed three. . .

DETD It was also established that **CpG**-stimulated mRNA expression requires endosomal acidification and NFκB activation in B cells and monocytes. J774 cells (2×10^6 cells/ml) were cultured for. . . stimulated with the addition of E. coli DNA (EC: 50 μg/ml), calf thymus DNA (CT: 50 μg/ml), LPS (10 μg/ml), **CpG** ODN (1826; 1 μM), or control non **CpG** ODN (1911; 1 μM) for 3 hr. WEHI-231 B cells (5×10^5 cells/ml) were cultured in the presence or absence of gliotoxin (0.1 μg/ml) or bisgliotoxin (0.1 μg/ml) for 2 hrs and then stimulated with a **CpG** ODN (1826), or control non-**CpG** ODN (1911; TCCAGGACTTTCCTCAGGTT, SEQ. ID. NO. 97) at 0.5 μM for 8 hr. In both cases, cells were harvested and. . .

DETD The results indicate that leukocytes respond to **CpG** DNA through a novel pathway involving the pH-dependent generation of intracellular ROS. The pH dependent step may be the transport or processing of the **CpG** DNA, the ROS generation, or some other event. ROS are widely thought to be second messengers in signaling pathways in. . .

DETD Presumably, there is a protein in or near the endosomes that specifically recognizes DNA containing **CpG** motifs and leads to the generation of reactive oxygen species. To detect any protein in the cell cytoplasm that may specifically bind **CpG** DNA, electrophoretic mobility

shift assays (EMSA) were used with 5' radioactively labeled oligonucleotides with or without **CpG** motifs. A band was found that appears to represent a protein binding specifically to single stranded oligonucleotides that have **CpG** motifs, but not to oligonucleotides that lack **CpG** motifs or to oligonucleotides in which the **CpG** motif has been methylated. This binding activity is blocked if excess of oligonucleotides that contain the NFkB binding site was. . . suggests that an NFkB or related protein is a component of a protein or protein complex that binds the stimulatory **CpG** oligonucleotides.

DETD . . . points where NFkB was strongly activated. These data therefore do not provide proof the NFkB proteins actually bind to the **CpG** nucleic acids, but rather that the proteins are required in some way for the **CpG** activity. It is possible that a CREB/ATF or related protein may interact in some way with NFkB proteins or other proteins thus explaining the remarkable similarity in the binding motifs for CREB proteins and the optimal **CpG** motif. It remains possible that the oligos bind to a CREB/ATF or related protein, and that this leads to NFkB. . .

DETD Alternatively, it is very possible that the **CpG** nucleic acids may bind to one of the TRAF proteins that bind to the cytoplasmic region of CD40 and mediate. . .

DETD Method for Making **Immunostimulatory** Nucleic Acids

DETD . . . described (Uhlmann, E. And Peyman, A. (1990) Chem. Rev. 90:544; Goodchild, J. (1990) Bioconjugate Chem. 1:165). 2'-O-methyl nucleic acids with **CpG** motifs also cause immune activation, as do ethoxy-modified **CpG** nucleic acids. In fact, no backbone modifications have been found that completely abolish the **CpG** effect, although it is greatly reduced by replacing the C with a 5-methyl C.

DETD Therapeutic Uses of **Immunostimulatory** Nucleic Acid Molecules

DETD Based on their **immunostimulatory** properties, nucleic acid molecules containing at least one unmethylated **CpG** dinucleotide can be administered to a subject in vivo to treat an "immune system deficiency". Alternatively, nucleic acid molecules containing at least one unmethylated **CpG** dinucleotide can be contacted with lymphocytes (e.g. B cells, monocytic cells or NK cells) obtained from a subject having an. . .

DETD As reported herein, in response to unmethylated **CpG** containing nucleic acid molecules, an increased number of spleen cells secrete IL-6, IL-12, IFNγ, IFN-α, IFN-β, IL-1, IL-3, IL-10, TNF-α,. .

.

DETD **Immunostimulatory** nucleic acid molecules can also be administered to a subject in conjunction with a vaccine to boost a subject's immune system and thereby effect a better response from the vaccine. Preferably the **immunostimulatory** nucleic acid molecule is administered slightly before or at the same time as the vaccine. A conventional adjuvant may optionally. . .

DETD . . . antigen encoded by the vaccine determines the specificity of the immune response. Second, if the backbone of the plasmid contains **CpG** motifs, its functions as an adjuvant for the vaccine. Thus, **CpG** DNA acts as an effective "danger signal" and causes the immune system to respond vigorously to new antigens in the area. This mode of action presumably results primarily from the stimulatory local effects of **CpG** DNA on dendritic cells and other "professional" antigen presenting cells, as well as from the co-stimulatory effects on B cells.

DETD **Immunostimulatory** oligonucleotides and unmethylated **CpG** containing vaccines, which directly activate lymphocytes and co-stimulate an antigen-specific response, are fundamentally different from conventional adjuvants (e.g aluminum precipates),. . .

DETD In addition, an **immunostimulatory** oligonucleotide can be administered prior to along with or after administration of a chemotherapy or immunotherapy to increase the responsiveness. . . chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF. **CpG** nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Induction of NK activity and. . .

DETD Another use of the described **immunostimulatory** nucleic acid molecules is in desensitization therapy for allergies, which are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated **CpG** nucleic acids are predominantly of a class called "Th1" which is most marked; by a cellular immune response and is. . . are mediated by Th2 type immune responses and autoimmune diseases by Th1 immune response. Based on the ability of the **immunostimulatory** nucleic acid molecules to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of an **immunostimulatory** nucleic acid (or a vector containing a nucleic acid) alone or in conjunction with an allergen can be administered to. . .

DETD Nucleic acids containing unmethylated **CpG** motifs may also have

significant therapeutic utility in the treatment of asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated. . .

DETD As described in detail in the following Example 12, oligonucleotides containing an unmethylated **CpG** motif (I, e,. **TCCATGACGTTCCTGACGTT**; SEQ ID NO. 10) but not a control oligonucleotide (TCCATGAGCTTCCTGAGTCT; SEQ ID NO. 8) prevented the development of an inflammatory. . .

DETD For use in therapy, an effective amount of an appropriate **immunostimulatory** nucleic acid molecule alone or formulated as a delivery complex can be administered to a subject by any mode allowing. . .

DETD . . . to realize a desired biologic effect. For example, an effective amount of a nucleic acid containing at least one unmethylated **CpG** for treating an immune system deficiency could be that amount necessary to eliminate a tumor, cancer, or bacterial, viral or. . . such factors as the disease or condition being treated, the particular nucleic acid being administered (e.g the number of unmethylated **CpG** motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or. . .

DETD . . . Table 1). In some experiments, culture supernatants were assayed for IgM by ELISA, and showed similar increased in response to **CpG**-ODN.

DETD . . . C57BL/6 spleen cells were cultured in two ml RPMI (supplemented as described for Example 1) with or without 40 μM **CpG** or non-**CpG** ODN for forty-eight hours. Cells were washed, and then used as effector cells in a short term ^{51}Cr release assay. . .

DETD B cells were cultured with phosphorothioate ODN with the sequence of control ODN 1a or the **CpG** ODN 1d and 3Db and then either pulsed after 20 hr with ^{3H} uridine or after 44 hr with ^{3H}. . .

DETD . . . for 1 hr. At 37° C. in the presence or absence of LPS or the control ODN 1a or the **CpG** ODN 1d and 3Db before addition of anti-IgM (1 μ/ml). Cells were cultured for a further 20 hr. Before a. . .

DETD DBA/2 female mice (2 mos. old) were injected IP with 500 g **CpG** or control phosphorothioate ODN. At various time points after injection, the mice were bled. Two mice were studied for each. . .

DETD . . . 1(2U/ug of DNA) at 37° C. for 2 hr in 1×SSC with 5 mM MgCl2. To methylate the cytosine in **CpG** dinucleotide in E. coli DNA, E. coli DNA was treated with **CpG** methylase (M. SssI; 2U/μg of DNA) in NEBuffer 2 supplemented with 160 μM S-adenosyl methionine and incubated overnight at 37°. . .

DETD . . . humidifier incubator maintained in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 1.5 mM L-glutamine, 50 μ/ml), **CpG** or non-**CpG** phosphodiester ODN (O--ODN) (20 μM), phosphorothioate ODN (S--ODN) (0.5 μM), or E. coli or calf thymus DNA (50 μg/ml) at. . . IgM production). Concentrations of stimulants were chosen based on preliminary studies with titrations. In some cases, cells were treated with CpG O--ODN along with various concentrations (1-10 μg/ml) of neutralizing rat IgG1 antibody against murine IL-6 (hybridoma MP5-20F3) or control rat. . .

DETD . . . injected intravenously (iv) with PBS, calf thymus DNA (200 μg/100 μl PBS/mouse), E. coli DNA (200 μg/100 μl PBS/mouse), or **CpG** or non-**CpG** S--ODN (200 μg/100 μl PBS/mouse). Mice (two/group) were bled by retroorbital puncture and sacrificed by cervical dislocation at various time. . .

DETD Cell Proliferation assay. DBA/2 mice spleen B cells (5×10^4 cells/100 μl/well) were treated with media, **CpG** or non-**CpG** S--ODN (0.5 μM) or O--ODN (20 μM) for 24 hr at 37° C. Cells were pulsed for the last four. . .

DETD . . . a receptor-dependent mechanism. J. Clin. Invest. 93:944) at 250 mV and 960 μF. Cells were stimulated with various concentrations of **CpG** or non-**CpG** ODN after electroporation. Chloramphenicol acetyltransferase (CAT) activity was measured by a solution assay (Seed, B. and J. Y. Sheen (1988). . .

DETD Oligodeoxynucleotide Modifications Determine the Magnitude of B Cell Stimulation by **CpG** Motifs

DETD . . . central linkages are phosphodiester, but the two 5' and five 3' linkages are methylphosphonate modified. The ODN sequences studied (with **CpG** dinucleotides indicated by underlining) include:

DETD These sequences are representative of literally hundreds of **CpG** and non-**CpG** ODN that have been tested in the course of these studies.

DETD . . . (1990) J. Immunol 145:1039; and Ballas, Z. K. and W. Rasmussen (1193) J. Immunol, 150:17), with medium alone or with **CpG** or non-**CpG** ODN at the indicated concentrations, or with E. coli or calf thymus (50 μg/ml) at 37° C. for 24 hr.. . .

DETD . . . mice were then treated with oligonucleotides (30 μg in 200 μl saline by i.p. injection), which either contained an unmethylated **CpG** motif (i e., **TCCATGACGTTCCTGACGTT**; SEQ ID NO.10) or did the (i.e., control, TCCATGAGCTTCCTGAGTCT; SEQ ID NO. 8). Soluble SeEA (10 μg in 25 μl. . .

DETD . . . inflammatory cells are present in the lungs. However, when the mice are initially given a nucleic acid containing an unmethylated **CpG** motif along with the eggs, the inflammatory cells in the lung are not increased by subsequent inhalation of the egg. . .

DETD . . . inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a **CpG** oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes. . .

DETD FIG. 14 shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can actually redirect the cytokine response of the lung to production of II-12, indicating the Th1 type of immune. .

DETD FIG. 15 shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune. .

DETD **CpG** Oligonucleotides Induce Human PBMC to Secrete Cytokines

DETD . . . by standard centrifugation over Ficoll hypaque. Cells ($5\times10^{5/ml}$) were cultured in 10% autologous serum in 95 well microtiter plates with **CpG** or control oligodeoxynucleotides (24 μg/ml for phosphodiester oligonucleotides; 6 g/ml for nuclease resistant phosphorothioate oligonucleotides) for 4 hr in the. . .

1. A method for increasing the responsiveness of a cancer cell to a cancer therapy using an **immunostimulatory** nucleic acid as compared to the absence of the **immunostimulatory** nucleic acid, comprising: administering to a subject having a cancer an effective amount for increasing the responsiveness of a cancer cell to a cancer therapy of an **immunostimulatory** nucleic acid, comprising: $5'X_{1X2CGX3X43}'$ wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides, and wherein the sequence is not. . .

. . . claim 1, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

25. A method for enhancing recovery of bone marrow using an **immunostimulatory** nucleic acid as compared to the absence of the **immunostimulatory** nucleic acid in a subject undergoing or having undergone cancer therapy, comprising: administering to a subject undergoing or having undergone cancer therapy which damages the bone marrow an effective amount for enhancing the recovery of bone marrow of an **immunostimulatory** nucleic acid, comprising: $5'X_{1X2CGX3X43}'$ wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides.

. . . claim 25, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

30. In a method for stimulating an immune response in a subject having a cancer, the method of the type involving antigen dependent cellular cytotoxicity (ADCC), the improvement comprising: administering to the subject an **immunostimulatory** nucleic acid, comprising: $5'X_{1X2CGX3X43}'$ wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides.

. . .. claim 32, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

36. A method for treating or preventing cancer, comprising: administering to a subject having a cancer an effective amount for treating or preventing cancer of an **immunostimulatory** nucleic acid, comprising: $5'X_{1X2CGX3X43}'$ wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides, and wherein the sequence is not. . .

. . . claim 36, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

L15 ANSWER 4 OF 12 USPATFULL on STN
2002:194879 **Immunostimulatory** nucleic acid molecules for activating dendritic cells.
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Hartmann, Gunther, Munchen, GERMANY, FEDERAL REPUBLIC OF
University of Iowa Research Foundation, Iowa City, IA, United States (U.S. corporation)
US 6429199 B1 20020806
APPLICATION: US 1998-191170 19981113 (9)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed:

1. A method for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate a dendritic cell, wherein the method is performed ex vivo.

2. A method for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic cell, wherein the dendritic cell is an isolated dendritic cell.

3. The method of claim 1, wherein the isolated nucleic acid has a formula: 5'$N_{1X1CGX2N23}$' wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymine; X_2 is cytosine, adenine, or thymine; N is any nucleotide and N_1+N_2 is from about 0-25 nucleotides.

4. The method of claim 2, wherein the method is performed ex vivo.

5. The method of claim 4, further comprising contacting the dendritic cell with an antigen prior to the isolated nucleic acid.

6. A method for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic cell, wherein at least one nucleotide of the isolated nucleic acid has a phosphate backbone modification where in the method is peformed ex vivo.

7. The method of claim 6, wherein the phosphate backbone modification is a phosphorothioate or phosphorodithioate modification.

8. The method of claim 7, wherein the phosphate backbone modification occures at the 5' end of the nucleic acid.

9. The method of claim 8, wherein the nucleic acid backbone includes the phosphate backbone modification at the 5' intemucleotide linkages.

10. The method of claim 7, wherein the nucleic acid backbone includes the phosphate backbone modification at the 3' intemucleotide linkages.

11. The method of claim 10, wherein the phosphate backbone modification occurs at the last five internucleotide linkages of the 3' end of the nucleic acid.

12. The method of claim 1, wherein the isolated nucleic acid has a formula: 5'$N_{1X1X2CGX3X4N23}$' wherein at least one nucleotide separates consecutive CpGs; X_{1X2} is selected from the group consisting of TpT, CpT, TpC, and ApT; X_{3X4} is selected from the group consisting of GpT, GpA, ApA and ApT; N is any nucleotide and N_1+N_2 is from about 0-25 nucleotides.

13. A method for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide in an amount effective to activate the dendritic cell, wherein the isolated nucleic acid is selected from the group consisting of SEQ ID Nos. 84 and 85.

14. A method for cancer immunotherapy, comprising: administering an activated dendritic cell that expresses a specific cancer antigen to a subject having a cancer including the cancer antigen, wherein the activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG**

dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic cell.

15. A method for treating an infectious disease, comprising: administeri ng an activated dendritic cell that expresses a specific microbial antigen to a subject having an infection with a microorganism including the microbial antigen, wherein the activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic cell.

16. A method for treating an allergy, comprising: administering an activated dendritic cell that expresses a specific allergy causing antigen to a subject having an allergic reaction to the allergy causing antigen, wherein the activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic is from about 8-80 bases in length in an amount effective to activate the dendritic cell.

17. A method for generating a high yield of dendritic cells, comprising: administering an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective for activating dendritic cells to a subject; allowing the isolated nucleic acid to activate dendritic cells of the subject; and isolating dendritic cells from the subject.

18. A method for causing maturation of a dendritic cell, comprising contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to cause maturation of the dendritic cell.

19. A method for activating a dendritic cell, comprising: contacting a dendritic cell with an effective amount to activate a dendritic cell of an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide and an antigen.

20. The method of claim 19, wherein the dendritic cell is contacted with the antigen within 48 hours of contacting the dendritic cell with the isolated nucleic acid.

21. The method of claim 19, wherein the dendritic cell is contacted with the antigen within 24 hours of contacting the dendritic cell with the isolated nucleic acid.

TI **Immunostimulatory** nucleic acid molecules for activating dendritic cells
AI **US 1998-191170 19981113 (9)**
AB . . . activating dendritic cells. In particular, the invention relates to oligonucleotides which have a specific sequence including at least one unmethylated **CpG** dinucleotide which are useful for activating dendritic cells. The methods are useful for in vitro, ex-vivo, and in vivo methods. . .
SUMM . . . activating dendritic cells. In particular, the invention relates to oligonucleotides which have a specific sequence including at least one unmethylated **CpG** dinucleotide which are useful for activating dendritic cells.
SUMM . . . the vertebrate immune system has the ability to recognize the presence of bacterial DNA based on the recognition of so-called **CpG**-motifs, unmethylated cytidine-guanosine dinucleotides within specific patterns of flanking bases. According to these disclosures **CpG** functions as an adjuvant and is as potent at inducing B-cell and T-cell responses as the complete Freund's adjuvant, but is preferable since **CpG** induces a higher Th1 response and is less toxic. Alum, the adjuvant which is used routinely in human vaccination, induces the less favorable Th2 response. Compared to alum, **CpG** is a more effective adjuvant. The combination of **CpG** and alum was found to produce a synergistic adjuvant effect.
SUMM **CpG** oligonucleotides also show adjuvant effects towards various immune cells. For instance, **CpG** enhances the efficacy of monoclonal antibody therapy, thus functioning as an effective immune adjuvant for antigen immunization in a B cell lymphoma model. Cytotoxic T cell responses to protein antigen also are induced by **CpG**. Furthermore, the presence of **immunostimulatory** DNA sequences in plasmids was found to be necessary for effective intradermal gene immunization.
SUMM It was discovered according to an aspect of the invention that the adjuvant activity of **CpG** is based on the direct activation of dendritic cells by **CpG**. Potent **immunostimulatory CpG**

oligonucleotides and control oligonucleotides were found to cause dramatic changes in dendritic cells isolated from peripheral blood by immunomagnetic cell sorting. **CpG** oligonucleotides provided excellent Dendritic cell survival, differentiation, activation and maturation, and were superior to the combination of GM-CSF and LPS. In fact, the combination of **CpG** and GM-CSF produced unexpected synergistic effects on the activation of dendritic cells. The invention thus encompasses both **CpG** oligonucleotides and the combination of **CpG** oligonucleotides and cytokines such as GM-CSF as well as in vitro, ex vivo, and in vivo methods of activating dendritic. . .

SUMM . . . The method includes the steps of contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate a dendritic. . .

SUMM The isolated nucleic acid is one which contains at least one unmethylated **CpG** dinucleotide and which is from about 8-80 bases in length. In one embodiment the unmethylated **CpG** dinucleotide has a formula:

SUMM . . . cytosine, adenine, or thymine; N is any nucleotide and N_1+N_2 is from about 0-25 nucleotides. In another embodiment the unmethylated **CpG** dinucleotide has a formula:

SUMM . . . dendritic cell to an antigen; contacting the isolated dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the isolated nucleic acid is from about 8-80 bases in length; and allowing the isolated dendritic cell to. . .

SUMM The isolated nucleic acid is one which contains at least one unmethylated **CpG** dinucleotide and which is from about 8-80 bases in length. In one embodiment the unmethylated **CpG** dinucleotide has a formula:

SUMM . . . cytosine, adenine, or thymine; N is any nucleotide and N_1+N_2 is from about 0-25 nucleotides. In another embodiment the unmethylated **CpG** dinucleotide has a formula:

SUMM . . . including an effective amount for synergistically activating a dendritic cell of an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length; and an effective amount for synergistically activating a. . .

SUMM . . . assay includes the following steps: contacting an immature dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length; exposing the dendritic cell to a putative drug;. . .

SUMM . . . yield of dendritic cells. The method includes the following steps administering an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective for activating dendritic cells. . .

SUMM . . . The method includes the following steps: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to produce a CD40. . .

SUMM . . . The method includes the step of contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to cause maturation of. . .

DRWD FIG. 1 shows FACS chart depicting **CpG** oligonucleotide promoted survival of dendritic precursor cells. Freshly isolated dendritic precursor cells were incubated for 2 days in the presence. . . of either oligonucleotides or GMCSF (800 U/ml). Flow cytometric analysis of morphology (forward scatter, FSC; sideward scatter, SSC) showed that **CpG** oligonucleotides (2006: **CpG** phosphorothioate oligonucleotide, 1×2 μg/ml, 2080 **CpG** phosphodiester oligonucleotide, 3×30 μg/ml) promote survival of dendritic precursor cells, while the non **CpG** controls (2117: 2006 with methylated **CpG**; 2078: identical to 2080 but GpCs instead of CpGs) showed no positive effect on cell survival compared to the sample. . .

DRWD FIG. 2 is a graph showing that the combination of **CpG** and GMCSF enhances viability of dendritic cells. Dendritic precursor cells were isolated from peripheral blood and incubated for 48 hours with GMCSF (800 U/ml) and oligonucleotides (2006: **CpG** phosphorothioate; 2117: CpGs in 2006 methylated; 2 μg/ml) as indicated. Viability was examined by flow cytometry. Data represent the mean. . .

DRWD . . . as indicated and examined by flow cytometry (sideward scatter, SSC). Viable cells (2500 per sample) were counted. Phosphodiester oligonucleotides (2080: **CpG**; 2078: non-**CpG**) were added at 0 hours, 12 hours and 24 hours (30 μg/ml each time point).

DRWD FIG. 4 shows FACS charts demonstrating that ICAM-1 and MHC II expression of dendritic cells in response to GMCSF and **CpG**. Dendritic precursor cells were incubated for 48 hours in the presence of GMCSF (800 U/ml)

and 2006 (**CpG** phosphorothioate; 6 μg/ml). Expression of ICAM-1 (CD54) and MHC II was examined by flow cytometry (2500 viable cells are counted. . .

DRWD FIG. 5 is graphs depicting induction of co-stimulatory molecule expression on dendritic cells by **CpG**. Dendritic precursor cells were incubated for 48 hours in the presence of GMCSF (800 U/ml) and oligonucleotides (2006: **CpG** phosphorothioate, 6 μg/ml) as indicated. Expression of CD54 (ICAM-1) (panel A), CD86 (B7-2) (panel B) and CD40 (panel C) was. . .

DRWD FIG. 6 is graphs depicting the enhancement of CD40 expression on dendritic cells is **CpG** specific and not induced by LPS. Dendritic precursor cells are cultured for 48 hours in the presence of GMCSF (800 U/ml), LPS (10 ng/ml) and oligonucleotides (2006, **CpG** phosphorothioate, 6 μg/ml: 2117, methylated 2006; 2080 **CpG** phosphodiester, 30 μg/ml at 0 hours, 12 hours and 24 hours; 2078 GpC version of 2080). CD40 expression is examined. . . mean fluorescence intensity). Panel A and panel B show the results of two separate sets of experiments. Panel A shows **CpG** specificity (methylated control oligonucleotide) for the synergy of **CpG** and GMCSF for induction of CD40 expression. Panel B shows that **CpG** is equally effective in enhancing CD40 expression as GMCSF, and that this effect is **CpG** specific (GpC control oligonucleotide). Panel A and B represent the mean of two independent experiments each.

DRWD FIG. 7 is graphs depicting the induction of CD54 and CD86 expression on dendritic cells is **CpG** specific and not induced by LPS. Dendritic precursor cells are cultured for 48 hours in the presence of GMCSF (800 U/ml), LPS (10 ng/ml) and oligonucleotides (2006, **CpG** phosphorothioate, 2 μg/ml: 2117, methylated 2006). CD54 (panel A) and CD86 (panel B) expression is examined by flow cytometry (MFI,. . .

DRWD FIG. 8 shows FACS charts demonstrating that CD86 expression on monocyte-derived Dendritic cells is induced by LPS but not by **CpG**. CD14-positive monocytes were prepared from PBMC by immunomagnetic separation and incubated in the presence of GMCSF (800 U/ml) and IL-4. . . added. CD 86 expression is measured by flow cytometry (numbers represent mean fluorescence intensity). In this series of experiments, the non-**CpG** phosphorothioate control oligonucleotide 2041 (5'-CTG GTC TTT CTG GTT TTT TTC TGG-3') (SEQ ID NO: 93) was used. The results are representative for 8 independent experiments, in which **CpG** did not stimulate monocyte-derived dendritic cells.

DRWD FIG. 9 shows FACS charts demonstrating that **CpG** induces maturation (CD83 expression) of dendritic cells. After 48 hours incubation with GMCSF (800 U/ml), LPS (10 ng/ml) and oligonucleotides (2006: **CpG** phosphorothioate; 2117 methylated 2006; 2 μg/ml), CD83 and CD86 expression on dendritic cells is determined in flow cytometry. Values (%). . .

DRWD FIG. 10 are electron micrographs depicting **CpG** induction of morphologic changes in dendritic cells. Dendritic cells were incubated for 2 days in the presence of GMCSF (800. . .

DRWD FIG. 11 are electron micrographs depicting Ultrastructural differences due to **CpG** Dendritic cells were incubated for 2 days in the presence of GMCSF (800 U/ml) and 2006 (2 μg/ml) (panel A) or with GMCSF (800 U/ml) (panel B) and transmission electron microscopy was performed. In the presence of **CpG** (panel A) multilamellar bodies (>) and multivesicular structures can be seen.

DRWD FIG. 12 are electron micrographs depicting High magnification of **CpG**-characteristic ultrastructural differences. Dendritic cells incubated with GMCSF (800 U/ml) and 2006 (2 μg/ml) were examined by transmission electron microscopy. Arrows. . .

DETD . . . receptors which detect microbial molecules like LPS in their local environment. It has been discovered according to the invention that **CpG** has the unique capability to promote cell survival, differentiation, activation and maturation of dendritic cells. In fact dendritic precursor cells. . . a two day incubation with GM-CSF. Without GM-CSF these cells undergo apoptosis. It was discovered according to the invention that **CpG** was superior to GM-CSF in promoting survival and differentiation of dendritic cells (MHC II expression, cell size, granularity). As shown in the Examples below, the **CpG** phosphorothioate oligonucleotide 2006 (2 μg/ml) induced the expression of ICAM-1 (CD54) by 3.6-fold (p=0.02; n=5), the co-stimulatory molecule B7-2 (CD86). . . either GM-CSF alone or GM-CSF combined with LPS. Electron microscopy revealed major ultrastructural changes of dendritic cells in response to **CpG**, indicating that these cells were differentiated. Additionally **CpG** was found to induce maturation of dendritic cells. **CpG** oligonucleotide 2006 was superior to GM-CSF and LPS at inducing maturation marker CD83. A synergistic maturation effect was observed when **CpG** oligonucleotide 2006 and GM-CSF were used together.

DETD All effects of **CpG** on dendritic cells were **CpG**-specific as shown by control oligonucleotides with methylated **CpG** motifs and

oligonucleotides containing GpC instead of **CpG**. Thus, the addition of a **CpG** oligonucleotide is sufficient for improving survival, differentiation, activation and maturation of human dendritic cells. Since dendritic cells form the link between the innate and the acquired immune system the ability to activate dendritic cells with **CpG** supports the use of **CpG**-based strategies for immunotherapy against disorders such as cancer and allergic or infectious diseases.

DETD . . . and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor, N.Y., current edition). It is shown according to the invention that **CpG** functions as an adjuvant by activating dendritic cells. **CpG** is a particularly useful adjuvant in humans because of its low toxicity. Many potent adjuvants in mice or other animals,. . . like the Freunds complete adjuvant, cannot be used in humans due to toxicity. Additionally, as demonstrated in the examples below, **CpG** activates and matures human primary blood dendritic cells where other known adjuvants such as LPS fail to do so. Furthermore, **CpG** is known to induce a Th1 immune response which is believed to be superior to the immune response induced by. . .

DETD Thus the use of **CpG** allows the generation of mature dendritic cells from peripheral blood within two days in a well defined system. The application of **CpG** for this purpose is superior to GM-CSF, which is currently used for this purpose. **CpG** oligonucleotides have a longer half life, are less expensive, and show a greater magnitude of immune effects. The combination of **CpG** and GM-CSF shows synergistic activity for the induction of co-stimulatory molecules (CD86, CD40).

DETD . . . activating dendritic cells for in vitro, ex vivo and in vivo purposes. It was demonstrated according to the invention that **CpG** oligodeoxyribonucleotides are potent activators of dendritic cells. Dendritic cells are believed to be essential for the initiation of primary immune responses in immune cells in vivo. It was discovered, according to the invention, that **CpG** oligodeoxyribonucleotide was capable of activating dendritic cells to initiate primary immune responses in T cells, similar to an adjuvant. It was also discovered the **CpG** ODN induces the production of large amounts of IL-12 in dendritic cells, indicating its propensity to augment the development of Th1 immune responses in vivo. The findings that **CpG** oligonucleotides were sufficient for survival, differentiation, activation, and maturation of human dendritic cells demonstrate the potent adjuvant activity of **CpG** and provide the basis for the use of **CpG** oligodeoxyribonucleotides as immunotherapeutics in the treatment of disorders such as cancer, infectious diseases, and allergy. In one aspect, the invention. . . for activating a dendritic cell by contacting the dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide, wherein the nucleic acid is from about 8-80 bases in length.

DETD . . . to immunization. This is accomplished by contacting immature dendritic cells with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide to cause the dendritic cell to become activated and to mature. The activated dendritic cell is then incubated with. . .

DETD One specific use for the **CpG** nucleic acids of the invention is to activate dendritic cells for the purpose of enhancing a specific immune response against. . . active against a specific cancer antigen, the dendritic cells may be exposed to the cancer antigen in addition to the **CpG**. In other cases the dendritic cell may have already been exposed to antigen but may not be expressing the antigen. . . the invention may be performed by routine ex vivo manipulation steps known in the art, but with the use of **CpG** as the activator.

DETD The dendritic cells may also be contacted with **CpG** using in vivo methods. In order to accomplish this, **CpG** is administered directly to a subject in need of immunotherapy. The **CpG** may be administered in combination with an antigen or may be administered alone. In some embodiments, it is preferred that the **CpG** be administered in the local region of the tumor.

DETD The isolated dendritic cell is contacted with **CpG** and exposed to an antigen. Although either step may be performed first or the steps may be performed simultaneously, in one preferred embodiment the antigen is exposed to the immature dendritic cell before the cell is contacted with the **CpG**. It is believed that the antigen is taken up by the dendritic cell and then when the dendritic cell is contacted with the **CpG**, that the dendritic cell is activated to process and present the antigen. Preferably, the antigen is exposed to the cell within 48 hours of adding **CpG**. In a more preferred embodiment, the dendritic cell is exposed to the antigen within 24 hours of the **CpG**.

DETD . . . An infectious disease, as used herein, is a disease arising from the presence of a foreign microorganism in the body. **CpG** is used to stimulate an antigen specific dendritic cell which can activate a T cell response against an antigen of. . .

DETD . . . active disorders, the methods and products of the invention can

be used as a prophylactic vaccine. In this case, the **CpG** nucleic acid sequence is administered in vivo, preferably in the presence of an antigen or dendritic cells are prepared ex. . .

DETD The **CpG** oligonucleotides of the invention are **immunostimulatory** molecules. An "**immunostimulatory** nucleic acid molecule" refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (i.e. "**CpG** DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates (e.g. has a mitogenic effect on, or induces or increases cytokine expression by) a dendritic cell. An **immunostimulatory** nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have. . .

DETD In one preferred embodiment the invention provides an isolated **immunostimulatory** nucleic acid sequence containing a **CpG** motif represented by the formula:

DETD In another embodiment the invention provides an isolated **immunostimulatory** nucleic acid sequence containing a **CpG** motif represented by the formula:

DETD Preferably the **immunostimulatory** nucleic acid sequences of the invention include X_{1X2} selected from the group consisting of GpT, GpG, GpA and ApA and X_{3X4} is selected from the group consisting of TpT, CpT and GpT. For facilitating uptake into cells, **CpG** containing **immunostimulatory** nucleic acid molecules are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size (even many kb long) are **immunostimulatory** if sufficient **immunostimulatory** motifs are present, since larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not include. . . or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus mitogenic **CpG** motif is not a palindrome. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, where the oligonucleotide incorporates a phosphate backbone. . .

DETD Preferably the **immunostimulatory CpG** DNA is in the range of between 8 to 30 bases in size when it is an oligonucleotide. Alternatively, **CpG** dinucleotides can be produced on a large scale in plasmids, which after being administered to a subject are degraded into oligonucleotides. Preferred **immunostimulatory** nucleic acid molecules (e.g. for use in increasing the effectiveness of a vaccine or to treat an immune system deficiency. . .

DETD . . . in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated **CpG** containing nucleic acid molecules that are tens to hundreds of kbs long are relatively resistant to in vivo degradation. For shorter **immunostimulatory** nucleic acid molecules, secondary structure can stabilize and increase their effect. For example, if the 3' end of a nucleic. . .

DETD . . . modified backbone. It was shown according to the invention that modification of the oligonucleotide backbone provided enhanced activity of the **CpG** molecules of the invention when administered in vivo. **CpG** constructs, including at least two phosphorothioate linkages at the 5' end of the oligodeoxyribonucleotide and multiple phosphorothioate linkages at the. . .

DETD Both phosphorothioate and phosphodiester oligonucleotides containing **CpG** motifs were active in dendritic cells. However, based on the concentration needed to induce **CpG** specific effects, the nuclease resistant phosphorothioate backbone **CpG** oligonucleotides were more potent (2 μg/ml for the phosphorothioate vs. a total of 90 μg/ml for phosphodiester). In the concentration used in this study, phosphorothioate oligonucleotides without **CpG** motifs showed no background stimulatory activity such as that described earlier for high phosphorothioate oligonucleotide concentrations.

DETD . . . TCGTCGCTGTTGTCGTTTCTT (SEQ ID NO: 77), TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 84), TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO: 85) TGTCGTTGTCGTTGTCGTT (SEQ ID NO: 90), **TCCATGACGTTCCTGACGTT** (SEQ ID NO: 97), GTCG(T/C)T and TGTCG(T/C)T.

DETD The stimulation index of a particular **immunostimulatory CpG** DNA can be tested in various immune cell assays. Preferably, the stimulation index of the **immunostimulatory** CgG DNA with regard to B cell proliferation is at least about 5, preferably at least about 10, more preferably. . . treat an immune system deficiency by stimulating a cell-mediated (local) immune response in a subject, it is important that the **immunostimulatory CpG** DNA be capable of effectively inducing cytokine secretion by dendritic cells.

DETD Preferred **immunostimulatory CpG** nucleic acids should effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml. . . IL-6, 200 pg/ml IL-12, depending on the therapeutic indication, as determined by the assays described in the Examples. Other preferred **immunostimulatory CpG** DNAs should effect at least about

10%, more preferably at least about 15% and most preferably at least about 20%. . .

DETD . . . found that the motifs that stimulate murine cells best differ from those that are more effective with human cells. Certain **CpG** oligodeoxynucleotides are poor at activating human cells (oligodeoxyribonucleotide 1707, 1708, which contain the palindrome forming sequences GACGTC and CACGTG, respectively).

DETD The **CpG** oligonucleotides are used to induce survival, activation, maturation, and differentiation of dendritic cells. A dendritic cell has its ordinary meaning. . .

DETD . . . to the invention may be isolated from any source as long as the cell is capable of being activated by **CpG** to produce an active antigen expressing dendritic cell. Several in vivo sources of immature dendritic cells may be used according. . . marrow dendritic cells and peripheral blood dendritic cells are both excellent sources of immature dendritic cells that are activated by **CpG**. Other sources may easily be determined by those of skill in the art without requiring undue experimentation, by for instance, isolating a primary source of dendritic cells and testing activation by **CpG** in vitro (e.g., using assays described in the Examples section). The invention also encompasses the use of any immature dendritic cells maintained in culture as a cell line as long as the cell is capable of being activated by **CpG**. Such cell types may be routinely identified using standard assays known in the art.

DETD . . . that are known to be activated by cytokines to produce antigen presenting dendritic cells are capable of being activated by **CpG**. For instance, monocyte-derived dendritic cells are not activated by **CpG**. Recently, the method of monocyte-derived dendritic cells has attracted major attention because the incubation of purified CD14-positive monocytes with GM-CSF. . . situation. Although these cells are highly responsive to LPS it was discovered that monocyte-derived Dendritic cells do not respond to **CpG** (see Examples). It was also demonstrated that human monocytes, while highly sensitive to LPS, show a minor and delayed response to **CpG**.

DETD Peripheral blood dendritic cells isolated by immunomagnetic cell sorting, which are activated by **CpG**, represent a more physiologic cell population of dendritic cells than monocyte derived dendritic cells. Immature dendritic cells comprise approximately 1-3%. . . flow cytometry. Freshly isolated dendritic cells cultured in the absence of GM-CSF rapidly undergo apoptosis. Strikingly, in the presence of **CpG** oligonucleotides without addition of GM-CSF, both cell survival and differentiation is markedly improved compared to GM-CSF. In the presence of **CpG**, dendritic cells form cell clusters which when examined by ultrastructural techniques such as electron microscopy revealed characteristic dense multilamellar intracytoplasmic. . . and had only minor cellular processes. In addition to promoting survival and differentiation of dendritic cells, a single addition of **CpG** oligonucleotide led to activation as represented by upregulation of the co-stimulatory molecules ICAM-1 (CD54), B7-2 (CD86) and CD40. The combination of **CpG** oligonucleotide and GM-CSF enhanced the expression of CD86 and CD40 synergistically, proving that activation is not due to **CpG**-induced GM-CSF.

DETD In addition to activating dendritic cells **CpG** was capable of causing maturation of the dendritic cells. Maturation is assessed by the appearance of CD83, a specific marker for mature human dendritic cells. The presence of **CpG** alone for two days was sufficient to cause maturation of a variable percentage of the cells and the combination of GM-CSF and **CpG** was found to act synergistically to cause maturation of an even greater number of cells.

DETD Each of the effects observed by culturing cells in the presence of **CpG**, improved survival, differentiation, activation and maturation of dendritic cells, were **CpG** specific since control oligonucleotides with methylated CpGs and oligonucleotides with GpC instead of CpGs were inactive. Additionally, **CpG** was superior to LPS in inducing both activation and maturation.

DETD . . . dendritic cells plays a key role for the induction of cytotoxic T-cells from naive T-cells. The profound changes observed in **CpG**-stimulated dendritic cells are similar to those seen after activation by CD40 Lanzavecchia A. Licence to kill. Nature 1998; 393: 413-414.. . . signal under physiologic circumstances. In addition to the data presented herein the data presented in the parent application indicate that **CpG** may be substitutes for CD40 ligation on dendritic cells. CD40 and **CpG** perform a number of parallel actions. First, **CpG** and CD40 both activate c-Jun NH2-terminal kinase and p38, but do not activate the extracellular receptor kinase in B cells. Second, CD40 and **CpG** are each sufficient to induce proliferation of B-cells. Finally, both CD40 and **CpG** activate NK cells in an IL-12 dependent manner.

DETD The ability of **CpG** to activate human dendritic cells differs from that of murine dendritic cells. It has also been discovered that **CpG**

upregulates MHC class II and co-stimulatory molecules on murine Langerhans cells. In another study similar changes were described for murine. . . bone marrow-derived Dendritic cells. Sparwasser T, et al. Eur J Immunol 1998; 28: 2045-2054. In both studies the efficacy of **CpG** to induce co-stimulatory molecules does not exceed the effects seen for LPS, to which monocytic cells are highly sensitive. Murine monocytes/macrophages are known to secrete high amounts of inflammatory cytokines in response to **CpG**. Since the murine cell preparation may include other myelomonocytic cells in the analysis as well a secondary indirect effect of **CpG** on Dendritic cells in these cell preparations may have contributed to the described activation of Dendritic cells.

DETD It has been shown according to the invention that purified human blood dendritic cells are highly sensitive to **CpG**, while their response to LPS is barely detectable. The LPS concentration used in this study (10 ng/ml) is 10-fold higher. . . In contrast to human macrophages, the low sensitivity of human blood dendritic cells to LPS and the high sensitivity to **CpG** is striking.

DETD Certain Unmethylated **CpG** Containing Nucleic Acids Were Initially Demonstrated to Have B Cell Stimulatory Activity as Shown In Vitro and In Vivo

DETD . . . the other nonstimulatory control oligodeoxyribonucleotide. In comparing these sequences, it was discovered that all of the four stimulatory oligodeoxyribonucleotide contained **CpG** dinucleotides that were in a different sequence context from the nonstimulatory control.

DETD To determine whether the **CpG** motif present in the stimulatory oligodeoxyribonucleotide was responsible for the observed stimulation, over 300 oligodeoxyribonucleotide ranging in length from 5 to 42 bases that contained methylated, unmethylated, or no **CpG** dinucleotides in various sequence contexts were synthesized. These oligodeoxyribonucleotide, including the two original "controls" (ODN 1 and 2) and two. . . then examined for in vitro effects on spleen cells (representative sequences are listed in Table 1). Several oligodeoxyribonucleotides that contained **CpG** dinucleotides induced B cell activation and IgM secretion; the magnitude of this stimulation typically could be increased by adding more **CpG** dinucleotides (Table 1; compare ODN 2 to 2a or 3D to 3Da and 3Db). Stimulation did not appear to result. . .

DETD Mitogenic oligodeoxyribonucleotide sequences uniformly became nonstimulatory if the **CpG** dinucleotide was mutated (Table 1; compare ODN 1 to 1a; 3D to 3Dc; 3M to 3Ma; and 4 to 4a) or if the cytosine of the **CpG** dinucleotide was replaced by 5-methylcytosine (Table 1; ODN 1b, 2b, 3Dd, and 3Mb). Partial methylation of **CpG** motifs caused a partial loss of stimulatory effect (compare 2a to 2c, Table 1). In contrast methylation of other cytosines did not reduce oligodeoxyribonucleotide activity (ODN 1c, 2d, 3De and 3Mc). These data confirmed that a CpG motif is the essential element present in oligodeoxyribonucleotide that activate B cells.

DETD In the course of these studies, it became clear that the bases flanking the **CpG** dinucleotide played an important role in determining the murine B cell activation induced by an oligodeoxyribonucleotide. The optimal stimulatory motif was determined to consist of a **CpG** flanked by two 5' purines (preferably a GpA dinucleotide) and two 3' pyrimidines (preferably a TpT or TpC dinucleotide). Mutations of oligodeoxyribonucleotide to bring the **CpG** motif closer to this ideal improved stimulation (e.g. Table 1, compare ODN 2 to 2e; 3M to 3Md) while mutations. . . Table 1, compare ODN 3D to 3Df; 4 to 4b, 4c and 4d). On the other hand, mutations outside the **CpG** motif did not reduce stimulation (e.g. Table 1, compare ODN 1 to 1d; 3D to 3Dg; 3M to 3Me). For. . .

DETD . . . 10 As. Th effect of the G-rich ends is cis; addition of an oligodeoxyribonucleotide with poly G ends but no **CpG** motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated **CpG** were found to be more **immunostimulatory**.

DETD . . . the subsequent fall in stimulation when purified B cells with or without anti-IgM (at a submitogenic dose) were cultured with **CpG** oligodeoxyribonucleotide, proliferation was found to synergistically increase about 10-fold by the two mitogens in combination after 48 hours. The magnitude. . .

DETD Cell cycle analysis was used to determine the proportion of B cells activated by **CpG**-oligodeoxyribonucleotide. **CpG**-oligodeoxyribonucleotide induced cycling in more than 95% of B cells. Splenic B lymphocytes sorted by flow cytometry into CD23-(marginal zone) and. . . as were both resting and activated populations of B cells isolated by fractionation over Percoll gradients. These studies demonstrated that **CpG**-oligodeoxyribonucleotide induce essentially all B cells to enter the cell cycle.

DETD **Immunostimulatory** Nucleic Acid Molecules Block Murine B Cell Apoptosis

DETD . . . are rescued from this growth arrest by certain stimuli such as

LPS and by the CD40 ligand. oligodeoxyribonucleotide containing the **CpG** motif were also found to protect WEHI-23 1 from anti-IgM induced growth arrest, indicating that accessory cell populations are not required for the effect. Subsequent work indicates that **CpG** oligodeoxyribonucleotide induce Bcl-x and myc expression, which may account for the protection from apoptosis. Also, **CpG** nucleic acids have been found to block apoptosis in human cells. This inhibition of apoptosis is important, since it should enhance and prolong immune activation by **CpG** DNA.

DETD Method for Making **Immunostimulatory** Nucleic Acids

DETD . . . described (Uhlmann, E. and Peyman, A., 1990, Chem Rev. 90:544; Goodchild, J., 1990, Bioconjugate Chem. 1:165). 2'-O-methyl nucleic acids with **CpG** motifs also cause immune activation, as do ethoxy-modified **CpG** nucleic acids. In fact, no backbone modifications have been found that completely abolish the **CpG** effect, although it is greatly reduced by replacing the C with a 5-methyl C.

DETD Based on their **immunostimulatory** properties, nucleic acid molecules containing at least one unmethylated **CpG** dinucleotide can be used as described in detail. The nucleic acid molecules may also be used as set forth herein. . .

DETD **Immunostimulatory** nucleic acid molecules can also be administered to a subject in conjunction with a vaccine to boost a subject's immune system and thereby effect a better response from the vaccine. Preferably the **immunostimulatory** nucleic acid molecule is administered slightly before or at the same time as the vaccine. A conventional adjuvant may optionally. . .

DETD . . . antigen encoded by the vaccine determines the specificity of the immune response. Second, if the backbone of the plasmid contains **CpG** motifs, it functions as an adjuvant for the vaccine. Thus, **CpG** DNA acts as an effective "danger signal" and causes the immune system to respond vigorously to new antigens in the area. This mode of action presumably results primarily from the stimulatory local effects of **CpG** DNA on dendritic cells and other "professional" antigen presenting cells, as well as from the co-stimulatory effects on B cells.

DETD **Immunostimulatory** oligonucleotides and unmethylated **CpG** containing vaccines, which directly activate lymphocytes and co-stimulate an antigen-specific response, are fundamentally different from conventional adjuvants (e.g. aluminum precipitates),. . .

DETD In addition, an **immunostimulatory** oligonucleotide can be administered prior to, along with or after administration of a chemotherapy or immunotherapy to increase the responsiveness. . . chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF. **CpG** nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Induction of NK activity and. . .

DETD Another use of the described **immunostimulatory** nucleic acid molecules is in desensitization therapy for allergies, which are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated **CpG** nucleic acids are predominantly of a class called "Th1" which is most marked by a cellular immune response and is. . . are mediated by Th2 type immune responses and autoimmune diseases by Th1 immune response. Based on the ability of the **immunostimulatory** nucleic acid molecules to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of an **immunostimulatory** nucleic acid (or a vector containing a nucleic acid) alone or in conjunction with an allergen can be administered to. . .

DETD Nucleic acids containing unmethylated **CpG** motifs may also have significant therapeutic utility in the treatment of asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated. . .

DETD As described in Co-pending parent patent application U.S. Ser. No. 08/960,774, oligonucleotides containing an unmethylated **CpG** motif (i.e. **TCCATGACGTTCCTGACGTT**; SEQ IN NO: 97), but not a control oligonucleotide (TCCATGAGCTTCCTGAGTCT; SEQ ID NO: 98) prevented the development of an inflammatory. . .

DETD For use in therapy, an effective amount of an appropriate **immunostimulatory** nucleic acid molecule alone or formulated as a delivery complex can be administered to a subject by any mode allowing. . .

DETD . . . to realize a desired biologic effect. For example, an effective amount of a nucleic acid containing at least one unmethylated **CpG** for treating an immune system deficiency could be that amount necessary to eliminate a tumor, cancer, or bacterial, viral or. . . such factors as the disease or condition being treated, the particular nucleic acid being administered (e.g. the number of unmethylated **CpG** motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or. . .

DETD The compositions of the invention, including activated dendritic cells,

isolated **CpG** nucleic acid molecules, cytokines, and mixtures thereof are administered in pharmaceutically acceptable compositions. The compositions may be administered by bolus. . .

DETD As reported herein, in response to unmethylated **CpG** containing nucleic acid molecules, an increased number of spleen cells secrete IL-6, IL-12, IFN-γ, IFN-α, IFN-β, IL-1, IL-3, IL-10, TNF-α,.

. .

DETD Systemic administration of **CpG** alone in some embodiments is useful for immunotherapy against antigens. Alternative agents like GM-CSF have a shorter half life, although their synergistic effects with **CpG** will likely make this combination useful. On the other hand, some activators of dendritic cells like LPS or inflammatory cytokines. . . systemic use for this purpose not practical. The present study provides the functional rationale and methods for the use of **CpG** for dendritic cell-based immunotherapeutic strategies against cancer and for its use as an adjuvant in humans.

DETD Systemically administered **CpG** oligonucleotides enhances the availability of immature and mature dendritic cells in the blood and in tissues.

DETD . . . also useful for in vitro screening assays. For instance, immature dendritic cells may be used in vitro to identify other **CpG** specific motifs which are useful for activating or causing maturation of dendritic cells. These motifs may then be used in. . . ex vivo for activating dendritic cells. Additionally, the same type of assay may be used to identify cytokines or other **immunostimulatory** molecules which may have synergistic adjuvant effects when combined with isolated **CpG** nucleic acid sequences of the invention.

DETD . . . maturation. The assay would involve the addition of a putative drug to a immature dendritic cell which is activated by **CpG**. If the putative drug prevents activation, then it may be a compound which is therapeutically capable of inhibiting activation or. . .

DETD . . . CD14, CD16, CD56) (O'Doherty U, et al., "Dendritic cells freshly isolated from human blood express CD4 and mature into typical **immunostimulatory** dendritic cells after culture in monocyte-conditioned medium", J Exp Med, 1993; 178: 1067-1076). Using these characteristics, dendritic cells can be. . .

DETD . . . optimal for immunotherapeutic purposes. We found that monocyte-derived dendritic cells are sensitive to LPS but surprisingly are not activated by **CpG** motifs (FIG. 8). It is believed that the inability of monocyte-derived DC to respond to **CpG** might be due to the unphysiologic methods by which these cells are prepared. Consequently, the effect of **CpG** oligonucleotides on primary peripheral blood DC was examined.

DETD **CpG** Substitutes for GMCSF for DC Survival

DETD . . . their ability to activate human B-cells and NK-cells, we selected particularly potent oligonucleotides as examples of a family of active **CpG**-containing oligonucleotides for the use in the present study. The **CpG** oligonucleotides used were: 2006 (24-mer), 5'-TCG TCG TTT TGT CGT TTT GTC GTT-3' (SEQ ID NO: 84), completely phosphorothioate-modified, and 2080 (20-mer), 5'-TCG TCG TTC CCC CCC CCC CC-3' (SEQ ID NO: 94), un-modified phosphodiester. The non-**CpG** control oligonucleotides used were: 2117 (24-mer), 5'-TQG TQG TTT TGT QGT TTT GTQ GTT-3' (SEQ ID NO: 95), Q=5 methyl. . .

DETD . . . the absence of GMCSF, DC undergo apoptosis during the first two days of cell culture. We examined the effect of **CpG** oligonucleotides on survival of DC in cell culture. Freshly isolated DC were incubated in the presence of GMCSF or oligonucleotides. . . the formation of cell clusters within one day for both the sample with GMCSF alone and the sample with the **CpG** phosphorothioate oligonucleotide 2006. While the size of the clusters was not different between these two samples, the DC incubated with. . . of mature dendritic cells. This difference was distinctive between GMCSF and 2006 samples by using light microscopy. Without GMCSF or **CpG**, no clusters could be found but there was an increasing number of non-viable cells as revealed by trypan blue staining. Viability of DC was quantified by flow cytometry (FIG. 1). Cell survival was dramatically improved in the presence of **CpG** motifs. This effect was found to be **CpG** specific for both phosphorothioate (2006, 2117) and phosphodiester (2080, 2078) oligonucleotides, since both non-**CpG** control oligonucleotides (2117: methylated version of 2006; 2078: CpGs in 2080 inverted to GpCs) showed no improved survival compared to. . .

DETD . . . μg/ml) cell survival was low and comparable to the sample with cells only (10.8+-5.2% and 7.4+-4.2%). These results show that **CpG** can substitute for GMCSF for promoting DC survival, and that the combination of both is favorable over each of them. . .

DETD Increased Size and Granularity of DC Induced by **CpG** is Associated with Enhanced Expression of MHC II

DETD Flow cytometric analysis suggested that differentiation of DC is enhanced by **CpG** and is associated with an increase of cell size (FSC)

and granularity (SSC) (FIG. 1). The surface expression of MHC. . . II) and examined by flow cytometry (2500 viable cells counted) (FIG. 3). In the sample with cells only or the non-**CpG** oligonucleotide (2078), a large immature population with low granularity (SSC) and lower MHC II expression was found (FIG. 3 region. . . and high expression of MHC II representing differentiated DC (FIG. 3, region B). The addition of either GMCSF or the **CpG** oligonucleotide 2080 enhanced both granularity and MHC II expression on a per cell basis (FIG. 3 left two panels). The **CpG** oligonucleotide 2080 showed a superior effect compared to GMCSF indicating that **CpG** promotes differentiation of DC in addition to an enhancement of cell survival.

DETD **CpG** Increases Co-stimulatory Molecules on DC

DETD . . . immune response by DC. Functional activation of DC requires by the expression of co-stimulatory molecules. We examined the effect of **CpG** on the expression of the intercellular adhesion molecule-1 (ICAM-1, CD54), and the co-stimulatory surface molecules B7-2 (CD86) and CD40. First,. . . 5, panel C) was quantified in flow cytometry by the mean fluorescence intensity (MFI) of viable DC. In all experiments, **CpG** was superior to GMCSF in enhancing expression of co-stimulatory molecules. Compared to the cells only sample, the **CpG** oligonucleotide 2006 enhanced the expression of CD54 (25.0+-5.7 vs. 7.0+-1.8; p=0.02, n=5), CD86(3.9+-0.8 vs. 1.6+-0.3; p=0.01; n=5) and CD40 (3.5+-1.0. . .

DETD . . . using 2117 (methylated version of 2006) and 2078 (GpC version of 2080). As shown in FIG. 6 for CD40, the non-**CpG** oligonucleotide 2117 showed no synergistic enhancement of CD40 expression when combined with GMCSF (FIG. 6 panel A). The non-**CpG** oligonucleotide 2078 alone did not induce CD40 compared to cells only (FIG. 6 B). Induction of CD86 (FIG. 7 panel A) and CD54 (FIG. 7 panel B) was also found to be **CpG** specific.

DETD . . . the maximal response in terms of cytokine production. Monocyte-derived DC are highly sensitive to LPS but do not respond to **CpG** suggesting major functional differences between monocyte-derived DC and DC isolated from peripheral blood (FIG. 8).

DETD **CpG** Induces Maturation (CD83 expression) of DC

DETD . . . Freshly isolated DC were incubated for 3 days with GMCSF, LPS or oligonucleotides. In the absence of either GMCSF or **CpG**, or with the methylated control oligonucleotide 2117 (2 μg/ml), survival of cells was poor. The remaining viable cells did not. . . 2006 even enhances CD83 expression synergistically (37%) (FIG. 9, left dot plot, upper row). This induction of CD83 expression was **CpG** specific as shown by the control oligonucleotide 2117 in combination with GMCSF (9.7%) (FIG. 9, right dot plot, upper row).. . .

DETD Ultrastructural Changes of DC in Response to **CpG**

DETD We examined DC by electron microscopy to detect ultrastructural differences due to **CpG**. In scanning electron microscopy (FIG. 10), DC cultivated with either GMCSF and **CpG** (FIG. 10 A) or with **CpG** alone (FIG. 10B) displayed a more irregular shape, longer veil processes and sheet-like projections, and more intercellular contacts than cells cultivated with GMCSF alone (FIG. 10C) or in combination with the non-**CpG** control oligonucleotide (FIG. 10D). Transmission electron microscopic imaging revealed striking differences between DC generated with GMCSF combined with **CpG** (FIG. 11A) and GMCSF alone (FIG. 11B). DC generated in the presence of **CpG** showed multilamellar intracytoplasmic bodies of high density (FIG. 11A, FIG. 12, indicated by >), which are absent without **CpG** (FIG. 11B). In addition, **CpG**-generated DC showed prominent multivesicular bodies (FIG. 11A, FIG. 12, indicated by >>), and a less heterochromatin in the nucleus. The. . .

. . . for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate a dendritic. . .

. . . for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic. . .

. . . for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic. . .

. . . for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide in an amount effective to activate the dendritic cell, wherein the isolated nucleic acid is selected from the group. . .

. . . activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic. . .

. . . activated dendritic cell is prepared by contacting a dendritic cell

with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic. . .

. . . activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic is from about 8-80 bases in length in an amount effective to activate the dendritic cell.

. . .

. . . method for generating a high yield of dendritic cells, comprising: administering an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective for activating dendritic cells. . .

. . . maturation of a dendritic cell, comprising contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to cause maturation of. . .

. . . cell with an effective amount to activate a dendritic cell of an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide and an antigen.

L15 ANSWER 5 OF 12 USPATFULL on STN

2002:143951 Use of nucleic acids containing unmethylated **CpG** dinucleotide as an adjuvant.

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US 6406705 B1 20020618

APPLICATION: US 1999-325193 19990603 (9)

PRIORITY: US 1997-40376P 19970310 (60)

DOCUMENT TYPE: Utility; GRANTED.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A composition of a synergistic combination of adjuvants, comprising: an effective amount for inducing a synergistic adjuvant response of a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant.

2. The composition of claim 1, wherein the non-nucleic acid is an adjuvant that creates a depo effect.

3. The composition of claim 2, wherein the adjuvant that creates a depo effect is selected from the group consisting of alum, emulsion based formulations, mineral oil, non-mineral oil, water-in-oil emulsions, water-in-oil-in-water emulsions, Seppic ISA series of Montanide adjuvants; MF-59; and PROVAX.

4. The composition of claim 1, wherein the non-nucleic acid adjuvant is an immune stimulating adjuvant.

5. The composition of claim 4, wherein the immune stimulating adjuvant is selected from the group consisting of saponins, PCPP polymer; derivatives of lipopolysaccharides, MPL, MDP, t-MDP, OM-174 and Leishmania elongation factor.

6. The composition of claim 1, wherein the non-nucleic acid adjuvant is an adjuvant that creates a depo effect and stimulates the immune system.

7. The composition of claim 6, wherein the adjuvant that creates a depo effect and stimulates the immune system is selected from the group consisting of ISCOMS, SB-AS2, AS2, SB-AS4, non-ionic block copolymers and SAF.

8. The composition of claim 1, wherein the composition also includes an antigen that is selected from the group consisting of peptides, polypeptides, cells, cell extracts, polysaccharides, polysaccharide conjugates, lipids, glycolipids, carbohydrates, viruses, viral extracts and antigens encoded within nucleic acids.

9. The composition of claim 8, wherein the antigen is derived from an infectious agent selected from the group consisting of a virus, bacterium, fungus and parasite.

10. The composition of claim 8, wherein the antigen is a tumor antigen.

11. The composition of claim 8, wherein the antigen is an allergen.

TI Use of nucleic acids containing unmethylated **CpG** dinucleotide as an adjuvant

AI US 1999-325193 19990603 (9)

AB The present invention relates generally to adjuvants, and in particular to methods and products utilizing a synergistic combination of **immunostimulatory** oligonucleotides having at least one unmethylated **CpG** dinucleotide (**CpG** ODN) and a non-nucleic acid adjuvant. Such combinations of adjuvants may be used with an antigen or alone. The present invention also relates to methods and products utilizing **immunostimulatory** oligonucleotides having at least one unmethylated **CpG** dinucleotide (**CpG** ODN) for induction of cellular immunity in infants.

SUMM . . . to adjuvants, and in particular to methods and products utilizing a synergistic combination of oligonucleotides having at least one unmethylated **CpG** dinucleotide (**CpG** ODN) and a non-nucleic acid adjuvant.

SUMM Bacterial DNA, but not vertebrate DNA, has direct **immunostimulatory** effects on peripheral blood mononuclear cells (PBMC) in vitro (Krieg et al., 1995). This lymphocyte activation is due to unmethylated **CpG** dinucleotides, which are present at the expected frequency in bacterial DNA ({fraction (1/16)}), but are under-represented (**CpG** suppression, {fraction (1/50)} to {fraction (1/60)}) and methylated in vertebrate DNA. Activation may also be triggered by addition of synthetic oligodeoxynucleotides (ODN) that contain an unmethylated **CpG** dinucleotide in a particular sequence context. It appears likely that the rapid immune activation in response to **CpG** DNA may have evolved as one component of the innate immune defense mechanisms that recognize structural patterns specific to microbial. . .

SUMM **CpG** DNA induces proliferation of almost all (>95%) B cells and increases immunoglobulin (Ig) secretion. This B cell activation by **CpG** DNA is T cell independent and antigen non-specific. However, B cell activation by low concentrations of **CpG** DNA has strong synergy with signals delivered through the B cell antigen receptor for both B cell proliferation and Ig. . . al., 1995). This strong synergy between the B cell signaling pathways triggered through the B cell antigen receptor and by **CpG** DNA promotes antigen specific immune responses. In addition to its direct effects on B cells, **CpG** DNA also directly activates monocytes, macrophages, and dendritic cells to secrete a variety of cytokines, including high levels of IL-12. . . activity (Klinman et al., 1996, supra; Cowdery et al., 1996, supra; Yamamoto et al., 1992; Ballas et al., 1996). Overall, **CpG** DNA induces a Th1 like pattern of cytokine production dominated by IL-12 and IFN-γ with little secretion of Th2 cytokines. . .

SUMM . . . and a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant, and wherein the combination of adjuvants is administered in an effective amount. . .

SUMM The **CpG** oligonucleotide and the non-nucleic acid adjuvant may be administered with any or all of the administrations of antigen. For instance. . . antigen. In some embodiments the subject is administered a priming dose of antigen and oligonucleotide containing at least one unmethylated **CpG** dinucleotide before the boost dose. In yet other embodiments the subject is administered a boost dose of antigen and oligonucleotide containing at least one unmethylated **CpG** dinucleotide after the priming dose.

SUMM . . . response a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant, and wherein the combination of adjuvants is administered in an effective amount. . . aspect, the same method is performed but the subject is an infant and the Th1 response can be induced using **CpG** DNA alone, or **CpG** DNA in combination with a non-nucleic acid adjuvant at the same or different site, at the same or different time.

SUMM . . . of a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant. The composition may also include at least one antigen, which may be. . .

SUMM . . . The method involves the step of administering to an infant an antigen and an oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant in an effective amount for inducing cell mediated immunity or Th1-like responses. . .

SUMM The **CpG** oligonucleotide may be administered with any or all of the

administrations of antigen. For instance the **CpG** oligonucleotide or the combination of adjuvants may be administered with a priming dose of antigen. In another embodiment the **CpG** oligonucleotide or the combination of adjuvants is administered with a boost dose of antigen. In some embodiments the subject is administered a priming dose of antigen and oligonucleotide containing at least one unmethylated **CpG** dinucleotide before the boost dose. In yet other embodiments the subject is administered a boost dose of antigen and oligonucleotide containing at least one unmethylated **CpG** dinucleotide after the priming dose.

SUMM . . . receiving an antigen and at least one non-nucleic acid adjuvant and at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide in order to induce a stronger Th1 immune response than either the adjuvant or oligonucleotide produces alone.

SUMM . . . administering to a subject at least one non-nucleic acid adjuvant and at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide in order to induce a Th1 innate immune response. For longer term protection, these adjuvants may be administered more than once. In another embodiment, **CpG** DNA may be used alone at one or more of the administrations.

SUMM In each of the above described embodiments a **CpG** oligonucleotide is used as an adjuvant. The oligonucleotide in one embodiment contains at least one unmethylated **CpG** dinucleotide having a sequence including at least the following formula:

SUMM In some embodiments X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA. Preferably X_{1X2} are GpA or GpT and X_{3X4} are TpT. In other preferred. . .

SUMM . . . mice immunized with 1 μg recombinant HBsAg protein alone, adsorbed onto alum (25 mg Al^{3+}/mg HBsAg), with 100 μg of **immunostimulatory CpG** ODN 1826, or with both alum and **CpG** ODN. Left panel: Each point represents the group mean (n=10) for titers of anti-HBs (total IgG) as determined in triplicate. . .

SUMM . . . 1 μg recombinant HBsAg protein, with or without alum, and with 0, 0.1, 1, 10, 100 or 500 μg of **CpG** ODN 1826 added. Each point represents the group mean (n=10) for anti-HBs titers (total IgG) as determined by end-point dilution. . .

SUMM . . . phosphorothioate backbone (S) or a chimeric of phosphodiester center regions and phosphorothioate ends (SOS). Most of the ODN contained 1-3 **CpG** motifs but some of the ODN were non-**CpG** controls (1911, 1982, 2041). Each point represents the group mean (n=5) for anti-HBs titers (total IgG) as determined by end-point. . .

SUMM . . . BALB/c mice immunized with 1 μg recombinant HBsAg protein with alum (25 mg Al^{3+}/mg HBs/Ag), with 10 μg of **CpG** ODN 1826, or with both alum and **CpG** ODN. Some animals were boosted with the same or a different formulation after 8 weeks. Each point represents the group. . .

SUMM . . . without adjuvant or with various adjuvants alone or in combination. The adjuvants were: alum (25 mg Al^{3+}/mg HBs/Ag), with **CpG** DNA (10 jig **CpG** ODN 1826), monophosphoryl lipid A (MPL, 50 μg) and Freund's complete adjuvant (mixed 1:1 v/v with HBsAg solution). Each point. . .

SUMM . . . IgG (end-point ELISA titer) produced at 4 weeks in BALB/c mice immunized with 1 μg of HBsAg with or without **CpG** and/or IFA (mineral oil mixed 1:1 v/v) or CFA (complete Freund's adjuvant mixed 1:1 v/v). The numbers above each bar. . .

SUMM . . . amount of total IgG produced at 4 weeks in BALB/c mice immunized with 1 μg of HBsAg with or without **CpG** and/or MPL (monophosphoryl lipid A, 50 μg) or alum. The numbers above each bar indicate the IgG2a:IgG1 ratio, with a. . .

SUMM . . . immunized with 10 μg HBsAg-expressing DNA vaccine (pCMV-S), or with recombinant HBsAg (1 μg) with alum (25 mg Al3+/mg HBsAg), **CpG** ODN 1826 (10 μg) or both alum and **CpG** ODN. Each point represents the proportion of mice responding, the numbers above the bars show the number of responding over. . .

SUMM . . . pCMV-S), or with 1 μg recombinant HBsAg protein alone, adsorbed onto alum (25 mg Al^{3+}/mg HBsAg), with 100 μg of **immunostimulatory CpG** ODN 1826, or with both alum and **CpG** ODN. Upper panel: Each point represents the group mean of animals that seroconverted (see FIG. 8 for numbers of animals). . .

SUMM . . . 7 days of age) with 1 μg recombinant HBsAg protein with alum (25 mg Al^{3+}/mg HBsAg), with 10 μg of **CpG** ODN 1826, or with both alum and **CpG** ODN. Each point represents the group mean (see FIG. 8 for numbers of animals) for anti-HBs titers (IgG1 and IgG2a. . .

SUMM . . . vaccine (10 μg recombinant HBsAg protein with alum, SmithKline Beecham biologicals, Rixensart, BE) or with Engerix-B plus 500 μg of **CpG** ODN 1968. Each point represents the group mean (n=5) for anti-HBs titers in milli-Intemational units/ml (mIU/ml). A titer of

10. . .

SUMM . . . in millilnternational Units per millilitre (mIU/ml) in orangutans immunized with 10 µg HBsAg with alum (like the HBV commercial vaccine), **CpG** oligonucleotides (**CpG** ODN 2006, 1 mg) or both alum and **CpG** ODN. The numbers above the bars show the number of animals with seroconversion (upper numbers, >1 mIU/ml) or with seroprotection. . .

DETD The invention in one aspect is based on the discovery that formulations containing combinations of **immunostimulatory CpG** oligonucleotides and non-nucleic acid adjuvants synergistically enhance immune responses to a given antigen. Different non-nucleic acid adjuvants used in combination. . .

DETD It has been discovered according to the invention that the combination of **immunostimulatory CpG** oligonucleotides and alum, MPL and other adjuvants results in a synergistic immune response. Compared with the recombinant hepatitis B surface. . . . vaccine alone, addition of alum increases the level of antibodies in mice against HBsAg (anti-HBs) about 7-fold whereas addition of **CpG** ODN increases them 32-fold. When **CpG** ODN and alum are used together, a 500-1000 times higher level of anti-HBs was observed, indicating a strong synergistic response.. . . immunization with HBsAg and alum resulted in a strong Th2-type response with almost all IgG being of the IgG1 isotype. **CpG** ODN induced a high proportion of IgG2a, indicative of a Th1-type response, even in the presence of alum. Furthermore, it. . . to the invention that in very young mice (7 day old), immune responses were induced by HBsAg with alum and **CpG** ODN but not with alum or **CpG** ODN alone. The antibodies produced with **CpG** ODN were predominantly of the IgG2a isotype, indicating a strong Th1-type response. This is remarkable considering the strong Th2 bias. . . antibodies. As well, Th1 responses are associated with cytotoxic T lymphocytes (CTL) that can attack and kill virus-infected cells. Indeed, **CpG** ODN, alone or in combination with alum induced good CTL activity in both adult and neonatal mice. These studies demonstrate that the addition of **CpG** ODN to protein or DNA vaccines in combination with other adjuvants is a valid new adjuvant approach to improve efficacy.

DETD . . . and a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant, and wherein the combination of adjuvants is administered in an effective amount. . .

DETD . . . kidney dialysis patients, alcoholics) the rate of non-response can approach 50%. As set forth in the Examples below, alum plus **CpG** ODN gave higher anti-HBs titers than alum alone in a strain of mice which has MHC-restricted hypo-responsiveness to HBsAg, thought to result in a failure to recognize T-helper epitopes. **CpG** ODN also overcame non-response in mice genetically incapable of providing T-help owing to an absence of class II MHC. Similar. . . vaccine with less than 10% achieving seroprotection after 2 doses, but that nearly 100% of animals responded with use of **CpG** oligonucleotides alone or combined with alum. The synergistic response was evident because antibody titers were much higher with **CpG** ODN plus alum than with **CpG** ODN alone or alum alone and were more than additive. These results support the proposition that **CpG** ODN drives the T cell independent activation of B cells. Thus in addition to providing a more effective and easier. . .

DETD . . . specific for the type of cancer to which the subject is at risk of developing and an adjuvant and a **CpG** oligonucleotide the subject may be able to kill of the cancer cells as they develop. If a tumor begins to. . .

DETD Allergies are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated **CpG** oligonucleotides are predominantly of a class called "Th1" which includes IL-12 and IFN-γ. In contrast, Th2 immune response are associated. . .

DETD Based on the ability of the **CpG** oligonucleotides to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of a **CpG** oligonucleotide can be administered to a subject to treat or prevent an allergy.

DETD Since Th1 responses are even more potent with **CpG** DNA combined with non-nucleic acid adjuvants, the combination of adjuvants of the present invention will have significant therapeutic utility in. . .

DETD . . . administered a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant. An oligonucleotide containing at least one unmethylated **CpG** dinucleotide is a nucleic acid molecule which contains an unmethylated cytosine-guanine dinucleotide sequence (i.e. "**CpG** DNA" or DNA containing a 5' cytosine followed by 3' guanosine and

linked by a phosphate bond) and activates the immune system. The **CpG** oligonucleotides can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have increased immune activity. The **CpG** oligonucleotides or combination of adjuvants can be used with or without antigen.

DETD . . . from existing nucleic acid sources (e.g. genomic or cDNA), but are preferably synthetic (e.g. produced by oligonucleotide synthesis). The entire **CpG** oligonucleotide can be unmethylated or portions may be unmethylated but at least the C of the 5' CG 3' must. . .

DETD In one preferred embodiment the invention provides a **CpG** oligonucleotide represented by at least the formula:

DETD In another embodiment the invention provides an isolated **CpG** oligonucleotide represented by at least the formula:

DETD . . . separates consecutive CpGs; X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA; N is any nucleotide and N_1 and N_2 are nucleic acid sequences composed. . . may have more influence on the biological activity or the kinetics of the biological activity. In another preferred embodiment the **CpG** oligonucleotide has the sequence 5'$TCN_{1TX1X2CGX3X43}$'.

DETD Preferably the **CpG** oligonucleotides of the invention include X_{1X2} selected from the group consisting of GpT, GpG, GpA and ApA and X_{3X4} is selected from the group consisting of TpT, CpT and GpT. For facilitating uptake into cells, **CpG** containing oligonucleotides are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size. . . than 8 nucleotides (even many kb long) are capable of inducing an immune response according to the invention if sufficient **immunostimulatory** motifs are present, since larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not include. . .

DETD Preferably the **CpG** oligonucleotide is in the range of between 8 and 100 and more preferably between 8 and 30 nucleotides in size. Alternatively, **CpG** oligonucleotides can be produced on a large scale in plasmids. These may be administered in plasmid form or alternatively they. . .

DETD The **CpG** oligonucleotide and immunopotentiating cytokine may be directly administered to the subject or may be administered in conjunction with a nucleic. . .

DETD . . . capable of forming the usual Watson-Crick base pairs. In vivo, such sequences may form double-stranded structures. In one embodiment the **CpG** oligonucleotide contains a palindromic sequence. A palindromic sequence used in this context refers to a palindrome in which the **CpG** is part of the palindrome, and preferably is the center of the palindrome. In another embodiment the **CpG** oligonucleotide is free of a palindrome. A **CpG** oligonucleotide that is free of a palindrome is one in which the **CpG** dinucleotide is not part of a palindrome. Such an oligonucleotide may include a palindrome in which the **CpG** is not part of the palindrome.

DETD . . . in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated **CpG** oligonucleotides that are tens to hundreds of kbs long are relatively resistant to in vivo degradation, particularly when in a double-stranded closed-circular form (i.e., a plamid). For shorter **CpG** oligonucleotides, secondary structure can stabilize and increase their effect. For example, if the 3' end of an oligonucleotide has self-complementarity. . .

DETD . . . invention have a modified backbone. It has been demonstrated that modification of the oligonucleotide backbone provides enhanced activity of the **CpG** oligonucleotides when administered in vivo. **CpG** constructs, including at least two phosphorothioate linkages at the 5' end of the oligonucleotide in multiple phosphorothioate linkages at the. . .

DETD Both phosphorothioate and phosphodiester oligonucleotides containing **CpG** motifs are active in immune cells. However, based on the concentration needed to induce **CpG** specific effects, the nuclease resistant phosphorothioate backbone **CpG** oligonucleotides are more potent (2 μg/ml for the phosphorothioate vs. a total of 90 μg/ml for phosphodiester).

DETD . . . 08/960,774, filed on Oct. 30, 1996 and Oct. 30, 1997 respectively. Exemplary sequences include but are not limited to those **immunostimulatory** sequences shown in Table 1.

DETD The stimulation index of a particular **immunostimulatory CpG** DNA can be tested in various immune cell assays. Preferably, the stimulation index of the **CpG** oligonucleotide with regard to B cell proliferation is at least about 5, preferably at least about 10, more preferably at. . . on Oct. 30, 1996 and Oct. 30, 1997 respectively. For use in vivo, for example, it is important that the **CpG** oligonucleotide and adjuvant

be capable of effectively inducing activation of Ig expressing B cells. Oligonucleotides which can accomplish this include,. . .

DETD The oligonucleotide containing at least one unmethylated **CpG** is used in combination with a non-nucleic acid adjuvant and an antigen to activate the immune response. A "non-nucleic acid adjuvant" is any molecule or compound except for the **CpG** oligonucleotides described herein which can stimulate the humoral and/or cellular immune response. Non-nucleic acid adjuvants include, for instance, adjuvants that. . . adjuvants that create a depo effect and stimulate the immune system. In infants, the oligonucleotide containing at least one unmethylated **CpG** is used alone or in combination with a non-nucleic acid adjuvant and an antigen to activate a cellular immune response.

DETD When the **CpG** oligonucleotide containing at least one unmethylated **CpG** is administered in conjunction with another adjuvant, the **CpG** oligonucleotide can be administered before, after, and/or simultaneously with the other adjuvant. For instance, the combination of adjuvants may be. . . risk of infection from being infected. In cases where the combination of adjuvants is given without antigen, with repeated administrations, **CpG** oligonucleotides or one of the components in the combination may be given alone for one or more of the administrations.

DETD The **CpG** oligonucleotide containing at least one unmethylated **CpG** can have an additional efficacy (e.g., antisense) in addition to its ability to enhance antigen-specific immune responses.

DETD In addition to the use of the combination of **CpG** oligonucleotides and non-nucleic acid adjuvants to induce an antigen specific immune response in humans, the methods of the preferred embodiments. . .

DETD . . . birds are housed in closed quarters, leading to the rapid spread of disease. Thus, it is desirable to administer the **CpG** oligonucleotide and the non-nucleic acid adjuvant of the invention to birds to enhance an antigen-specific immune response when antigen is present. The **CpG** oligonucleotide and the non-nucleic acid adjuvant of the invention could also be administered to birds without antigen to protect against. . .

DETD . . . may be administered subcutaneously, by spray, orally, intraocularly, intratracheally, nasally, in ovo or by other methods described herein. Thus, the **CpG** oligonucleotide and non-nucleic acid adjuvant of the invention can be administered to birds and other non-human vertebrates using routine vaccination. . .

DETD . . . the invention can be used to protect against infection in livestock, such as cows, horses, pigs, sheep, and goats. The **CpG** oligonucleotide and the non-nucleic acid adjuvant of the invention could also be administered with antigen for antigen-specific protection of long. . .

DETD . . . method for immunizing an infant by administering to an infant an antigen and an oligonucleotide containing at least one unmethylated **CpG** dinucleotide in an effective amount for inducing cell mediated immunity in the infant. In some embodiments the infant is also. . .

DETD . . . in 10-15% of individuals infected as adolescents or adults, but 90-95% for those infected (either vertically or horizontally) as infants. **CpG** oligonucleotides may be used, according to the invention, to reduce this further owing to a more rapid appearance and higher. . .

DETD . . . expression of a particular cytokine when higher levels are desired. Modulation of a particular cytokine can occur locally or systemically. **CpG** oligonucleotides can directly activate macrophages and dendritic cells to secrete cytokines. No direct activation of proliferation or cytokine secretion by. . . Cytokine profiles determine T cell regulatory and effector functions in immune responses. In general, Th1-type cytokines are induced, thus the **immunostimulatory** nucleic acids promote a Th1 type antigen-specific immune response including cytotoxic T-cells.

DETD . . . for inducing a Th1 immune response. The combination of adjuvants includes at least one oligonucleotide containing at least one umnethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant. It was not previously known that when **CpG** was combined with a non-nucleic acid adjuvant, as described above, that the combination would produce an immune response with a. . . by the combination of adjuvants is synergistic. Another aspect of the invention is to induce a Th response by using **CPG** with a non-nucleic acid adjuvant that by itself induces a Th2 response.

DETD . . . Adjuvants that induce Th1 responses include but are not limited to MPL, MDP, ISCOMS, IL-12, IFN-γ, and SB-AS2. When the **CpG** oligonucleotide is administered with a non-nucleic acid adjuvant the combination of adjuvants causes a commitment to a Th1 profile, that neither the adjuvant nor the **CpG** oligonucleotide is capable of producing on its own. Furthermore, if the non-nucleic acid adjuvant on its own induces a Th2 response, the addition of **CpG** oligonucleotide can overcome this Th2 bias and induce a Th1 response that may be even more Th1-like than with **CpG** alone.

DETD . . . Let. 29:2619-2622, 1988). These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, **CpG** dinucleotides can be produced on a large scale in plasmids, (see Sambrook, T., et al., Molecular Cloning: A Laboratory Manual,. . .

DETD Nucleic acids containing an appropriate unmethylated **CpG** can be effective in any mammal, preferably a human. Different nucleic acids containing an unmethylated **CpG** can cause optimal immune stimulation depending on the mammalian species. Thus an oligonucleotide causing optimal stimulation in humans may not. . .

DETD The **CpG** ODN of the invention stimulate cytokine production (e.g., IL-6, IL-1 2, IFN-γ, TNF-α and GM-CSF) and B-cell proliferation in PBMC's. . .

DETD . . . TGTCGTTGTCGTTGTCGTT; (SEQ ID NO: 82)
TCGTCGTCGTCGTT; (SEQ ID NO: 83)
TGTCGTTGTCGTT; (SEQ ID NO: 84)
TCCATAGCGTTCCTAGCGTT; (SEQ ID NO: 85)
TCCATGACGTTCCTGACGTT; (SEQ ID NO: 86)
GTCGYT; (SEQ ID NO: 87)
TGTCGYT; (SEQ ID NO: 88)
AGCTATGACGTTCCAAGG; (SEQ ID NO: 89)
TCCATGACGTTCCTGACGTT; (SEQ ID NO: 90)
ATCGACTCTCGAACGTTCTC; (SEQ ID NO: 91)
TCCATGTCGGTCCTGACGCA; (SEQ ID NO: 92)
TCTTCGAT; (SEQ ID NO: 93)
ATAGGAGGTCCAACGTTCTC;. . .

DETD Preferred **CpG** ODN can effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml of. . . indication. These cytokines can be measured by assays well known in the art. The oligonucleotides listed above or other preferred **CpG** ODN can effect at least about 10%, more preferably at least about 15% and most preferably at least about 20%. . .

DETD The term "effective amount" of a **CpG** oligonucleotide refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of an oligonucleotide containing at least one unmethylated **CpG** and a non-nucleic acid adjuvant for treating an infectious disorder is that amount necessary to cause the development of an. . . amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular **CpG** oligonucleotide being administered (e.g. the number of unmethylated **CpG** motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or. . .

DETD The use of **CpG** ODN as an adjuvant alone or in combination with other adjuvants was evaluated. The hepatitis B virus surface antigen (HBsAg). . .

DETD . . . cells (Medix Biotech #ABH0905). This was diluted in saline for use without adjuvant. HBsAg was also formulated with alum and/or **CpG** ODN as adjuvant. HBsAg protein was mixed with aluminum hydroxide (Alhydrogel 85, [Al_{2O3}], Superfos Biosector, Vedbaek, Denmark) in the same. . .

DETD For groups treated with **CpG** ODN, an appropriate volume of synthetic oligodeoxynucleotide (ODN # 1826) of the sequence **TCCATGACGTTCCTGACGTT** (SEQ ID NO. 86) synthesized with a phosphorothioate backbone (Oligos Etc. & Oligo Therapeutics, Wilsonville, Oreg.) was added alone or. . . injection into the left tibialis anterior (TA) muscle of 1 or 2 ug HBsAg, without or with adjuvant (alum and/or **CpG** ODN), in 50 1 vehicle. When **CpG** DNA was added, each animal received a total of 1, 10, 100 or 500 μg ODN. Newborn mice were immunized. . .

DETD . . . mg Al3+/mg HbsAg). Each monkey received an injection of 0.5 ml containing 10 μg HbsAg. For some monkeys, 500 μg **CpG** ODN 1968 (TCGTCGCTGTTGTCGTTTCTT) (SEQ ID NO 72) was added to the vaccine formulation.

DETD . . . into the anterior thigh muscle of HBsAg *ay subtype, 20 μg/ml) combined with alum (25 mg Al3+/mg HBsAg), combined with **CpG**. **CpG** ODN 2006 (TCGTCGTGTCGTGTCGTT) (SEQ ID NO 77) was added to the vaccine formulation. Each orangutan received an injection of 1.0 ml containing 20 μg HBsAg with alum (500 μg), **CpG** oligonucleotide (1 mg) or both adjuvants.

DETD Comparison of **CpG** ODN and Non-nucleic Acid Adjuvants with HBsAg Subunit Vaccine

DETD . . . (i) alone, (ii) mixed with alum, (iii, iv, v, vi, vii) mixed with 0.1, 1, 10, 100 or 500 μg **CpG** ODN, or (viii, ix, x, xi, xii) mixed with both alum and 0.1, 1, 10, 100 or 500 μg **CpG** ODN. These mice were bled at 1, 2, 4 and 8 weeks after immunization and the plasma was assayed for. . .

DETD . . . of mice (n=5) were immunized with HBsAg (1 μg) alone, with alum (25 μg Al3+), with one of several different **CpG** and non-**CpG** control oligonucleotides of different backbones (10 μg), or with both alum and an oligonucleotide.

DETD Other groups of mice (n=5) were immunized as above (except only the 10 µg dose of **CpG** ODN was used) and boosted with the identical or a different formulation at 8 weeks, then spleens were removed 2. . .

DETD . . . mice were immunized with HBsAg (1 µg) and one of the following non-nucleic acid adjuvants alone or in combination with **CpG** ODN (10 µg): monophosphoryl lipid A (MPL, 50 µg, Ribi); Freund's Complete Adjuvant (CFA; 1:1 v/v); Freund's Incomplete Adjuvant (IFA;. . .

DETD . . . 3, 7 or 14 days were injected with (i, ii, iii) a total of 1 µg HBsAg with alum, with **CpG** ODN 1826 (10 µg) or with both alum and **CpG** ODN, or with (iv) an HBsAg-expressing DNA vaccine (1-µg pCMV-S). Plasma was obtained at 4, 8, 12 and 16 weeks. . .

DETD Immunization of Cynomolgus Monkeys with HBsAg and Alum or Alum+**CpG** ODN

DETD . . . (HBsAg at 20 mg/ml adsorbed to alum, 25 mg Al3+/mg HBsAg) to which had been added saline (0.1 ml) or **CpG** ODN 2006 (500 µg in 0.1 ml, SEQ #77). Monkeys were bled at 2, 8, 10, 12 and 14 weeks. . .

DETD Immunization of Orangutans with HBsAg and Alum or **CpG** ODN or Alum+**CpG** ODN

DETD . . . and 4 weeks with 1 ml of vaccine containing HBsAg (10 µg) plus (i) alum (25 mg Al3+/mg HBsAg)(n=13), (ii) **CpG** ODN 2006 (SEQ# 77) (m=24) or (iii) alum plus **CpG** ODN (n=14). Animals were bled at 4.8 and 12 weeks and plasma was evaluated for anti-HBs titers (mIU/ml).

DETD Synergy of **CpG** ODN with Alum as Adjuvant for HBV Subunit Vaccine in Mice

DETD . . . (<100) by 4 weeks. These titers were about 10-fold higher with the addition of alum as adjuvant, 60-fold higher with **CpG** ODN and more than 500-fold higher with both alum and **CpG** ODN. At later time points, the highest peak titers were with HBsAg/alum/**CpG**, the second highest with HBsAg/**CpG**, then HBsAg/alum (FIG. 1).

DETD . . . the immune system is even less mature than a newborn human, 10% and 0% of mice seroconverted with alum and **CpG** ODN alone respectively, but 75% serocoinverted when **CpG** ODN and alum were used together. In 7 day old mice, which have an immune system similar in maturity to that of a newborn human, seroconversion for alum, **CpG** or the combination was 11%, 22% and 1 00% respectively (FIG. 8). Furthermore, in these 7 day old mice, antibody. . .

DETD When used alone or combined with alum, there is a dose-response for **CpG** ODN with the best results being obtained with an intermediate dose (10 µg) and no further or only relatively small. . .

DETD When a large panel of ODN is compared for adjuvant activity it can be seen that **CPG** ODN with a nuclease-resistant phosphorothioate backbone have the best adjuvant effects (FIG. 3). There was very little or no adjuvant activity of non-**CpG** control ODN with a phosphorothioate backbone; or of **CpG** ODN with a chimeric or phosphodiester backbone. However, for those phosphorothioate **CpG** ODN that did not have adjuvant effect, all exhibited a synergistic effect with alum. In general, antibody titers with combined alum and **CpG** ODN were 10 to 100-fold higher than with **CpG** ODN and/or 100 to 1000-fold higher than with alum alone (FIG. 3).

DETD . . . with HBsAg and no adjuvant, and were completely lost with the addition of alum. CTL were augmented equally with both **CpG** ODN as with combined alum and **CpG** ODN (FIG. 1). A synergy for CTL responses could be seen with prime-boost strategies, in that priming with **CpG** ODN and boosting with alum gave better CTL than priming and boosting with **CpG** alone (FIG. 4) (Note: use of alum alone completely abrogates the CTL response).

DETD A synergistic action of **CpG** ODN and alum on CTL was very evident with immunization of young (7 day old) mice. In this case, neither alum nor **CpG** ODN used alone induced significant levels of HBsAg-specific CTL, but when used together there wre very strong CTL were observed. . .

DETD Thus, **CpG** ODN is superior to alum for both humoral and cell-mediated responses, when each is used alone as adjuvant with the. . . action such that antibody and CTL activity are stronger than when either adjuvant is used alone. These results indicate that **CpG** ODN could be used to replace alum in vaccine formulations, which could be desirable to avoid associated side-effects due to. . . not possible to use alum because chemical interactions interfere with the efficacy of the vaccine. This should not occur with **CpG** ODN. Of even greater interest is the strong synergistic response when **CpG** ODN and alum are used together as adjuvants. This could allow better immune responses with lower or fewer doses of antigen. There is a fairly flat dose response to **CpG** ODN whether or not alum is present, indicating that a wide range of **CpG** ODN could be useful to adjuvant vaccines in humans.

DETD Synergy of **CpG** ODN with Other Non-nucleic Acid Adjuvants for HBV Subunit Vaccine in Mice.

DETD As discussed above, **CpG** ODN alone gave 8-fold higher antibody titers than alum, the only adjuvant currently licensed for human use. It also produces. . . in a dose of five times less than that of MPL. There was, as discussed above, a strong synergy with **CpG** ODN and alum, but in contrast no such synergy was seen with MPL and alum. Owing to the

strong synergistic effect of alum and **CpG** ODN, this combination of adjuvants is even better than Freund's complete adjuvant (FCA) for inducing antibodies in mice (FIG. 5). . .

DETD The synergy seen with **CpG** ODN and alum, was also seen with **CpG** ODN combined with other adjuvants. When used alone, **CpG** ODN and Freund's incomplete adjuvant (FIA, a type of mineral oil) induced similar antibody titers, but when used together the anti-HBs titers were more than 50-fold higher than with either adjuvant alone. Indeed, the combination of **CpG** ODN and FIA was even better than FCA (FIG. 6).

DETD Similarly, **CpG** ODN and MPL alone gave equally high antibody titers, but when used together the titers were about 4-times higher than with either adjuvant alone (FIG. 7). While the synergistic response with **CpG** and MPL was not as marked with respect to overall antibody titers, it was very pronounced with respect to the. . .

DETD Dominance and Synergy of **CpG** ODN with Alum for Induction of a Th1-type immune response including CTL

DETD . . . to Th1 -type cytokines such as IL-12 and IFN-γ. Rather, almost all (>99%) antibodies were of the IgG1 isotype IgG2a:IgG1=0.01. **CpG** ODN induces significantly more IgG2a antibodies, such that they made up at least 50% of the total IgG (IgG (IgG2a:IgG1=1.4). The combination of alum and **CpG** ODN induce an equally strong Th1 response as **CpG** ODN alone (IgG2a:IgG1=1.0), despite the extremely strong Th2-bias of alum (FIG. 5). Similarly CTL responses with **CpG** ODN plus alum were as strong as those with **CpG** ODN alone, despite the fact that the Th2-bias of alum resulted in a complete loss of CTL when alum was. . .

DETD The strong Th1 bias with **CpG** is even more evident in neonatal and young mice, which are known to naturally have a strong Th2-bias to their immune system. In this case, neither alum nor **CpG** ODN on their own induced detectable IgG2a, indicating a very poor or absent Th1 response. Remarkably, when used together, **CpG** ODN and alum induced high levels of Ig G2a antibodies, which were now the predominant form of IgG (FIG. 10). Similarly, neither **CpG** ODN or alum induced significant levels of CTL in young mice, yet when used together there was a strong CTL. . .

DETD The strength of the Th1 influence of **CpG** ODN is seen not only by its ability to dominate over the Th2 effect of alum when they are co-administered,. . . owing to the strong Th2 bias of alum (FIGS. 1 and 4). However, in mice using alum at prime and **CpG** at boost, good CTL were induced, indicating the possibility of **CpG** to overcome a previously established Th2 response (FIG. 4).

DETD . . . of HBV-specific CTL is thought to contribute to the chronic carrier state. In contrast, one of the primary advantages of **CpG** DNA over alum as an adjuvant is the Th1-bias of the responses and thus the possibility to induce CTL. A striking finding from the present study is that **CpG** can completely counteract the Th2-bias of alum when the two adjuvants are delivered together, and in the case of immunization in early life, the combination can even give a more Th1 response than **CpG** ODN alone. This could allow one to capitalize on the strong synergistic action of the two adjuvants on the humoral. . .

DETD . . . the high hygiene level and rapid treatment of childhood infections (Cookson and Moffatt, 1997). Early exposure to bacterial DNA (and **immunostimulatory CpG** motifs) pushes the immune system away from Th2- and towards a Th1-type response and this may account for the lower. . . asthma in less developed countries, where there is a much higher frequency of upper respiratory infections during childhood. Addition of **CpG** ODN as adjuvant to all pediatric vaccines could re-establish a Th1-type response thereby reducing the incidence of asthma.

DETD Synergy of **CpG** ODN with Other Adjuvants for Induction of a Th1-type Immune Responses

DETD The synergistic effect of **CpG** ODN on Th1 responses was also seen using other adjuvants. IFA on its own induces a very strong Th2-type response with virtually no IgG2a antibodies (IgG2a:IgG1=0.002) and **CpG** ODN on its own induces a moderate Th1 response (IgG2a:IgG1=1.4), but together the response was very strongly Th1 (IgG2a:IgG1=24.0). It. . .

DETD Similarly, **CpG** and MPL on their own are moderately Th1 (IgG2a:IgG1 ratios at 4 weeks are 1.4 and 1.9 respectively), but together. . .

DETD **CpG** ODN as Synergistic Adjuvant in Cynomolgus Monkeys

DETD **CpG** ODN, in combination with alum, also acts as a potent adjuvant to augment anti-HBs responses in Cynomolgus monkeys. Compared to responses obtained with the commercial HBV vaccine that contains alum, monkeys immunized with the commercial vaccine plus **CpG** ODN attained titers 50-times higher after prime and 10-times higher after boost (FIG. 14).

DETD **CpG** ODN as Synergistic Adjuvant to HBsAg in Hyporesponder Orangutans

DETD . . . 1988), only 0% and 15% of vaccinated orangutans have seroconverted by the same times. With the addition of 1 mg **CpG** ODN, this becomes 43% and 100% respectively. A synergistic response is seen even in these hyporesponders, because antibody levels and seroconversion rates are better with **CpG** ODN plus alum than with either adjuvant

alone (FIG. 12).

. . . of a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant.

L15 ANSWER 6 OF 12 USPATFULL on STN

2002:9854 Vectors and methods for immunization or therapeutic protocols.

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US 6339068 B1 20020115

APPLICATION: US 1998-82649 19980520 (9)

PRIORITY: US 1997-47209P 19970520 (60)
US 1997-47233P 19970520 (60)

DOCUMENT TYPE: Utility; GRANTED.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A method for producing an **immunostimulatory** nucleic acid construct comprising at least one **CpG**-S motif and a nucleic acid encoding an antigen comprising: determining **CpG**-N and **CpG**-S motifs present in a nucleic acid construct comprising at least one **CpG**-S motif and a nucleic acid encoding an antigen; removing **CpG**-N motifs from the nucleic acid construct; and optionally inserting **CpG**-S motifs into the nucleic acid construct, thereby producing said **immunostimulatory** nucleic acid construct that stimulates an immune response against the antigen, wherein the **CpG**-N motifs comprise motifs selected from the group consisting of direct repeats of **CpG** dinucleotides, CCG trinuclectides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides, a combination thereof, and a poly-G motif, wherein the poly-G motif optionally contains at least four Gs in a row or two G trimers, and wherein the **CpG**-S motifs comprise motifs having the formula 5'$X_1CGX_2$3' wherein C is unmethylated, at least one nucleotide separates consecutive CG dinucleotides in the motif, X_1 is selected from the group consisting of adenine, guanine, and thymine and X_2 is selected from the group consisting of cytosine, thymine, and adenine, and wherein the **CpG**-N motifs are removed from non-essential regions of the nucleic acid construct and the **CpG**-S motifs are inserted into non-essential regions of the nucleic acid construct, wherein the antigen is selected from the group consisting of a manmmalian antigen, an avian antigen, an antigen from a pathogen that infects mammalian and avian subjects, wherein the pathogen is selected from the group consisting of a bacterium, a virus, a fungus and a parasite.

2. The method of claim 1, wherein the **CpG**-N motifs are removed by site-specific mutagenesis.

3. The method of claim 1, wherein the **CpG**-N motifs are selected from the group consisting of clusters of direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucilcotides, CGCG tetranucleotides and a combination thereof.

4. The method of claim 1, wherein the nucleic acid construct is a plasmid.

5. The method of claim 1, wherein the nucleic acid construct is a viral vector.

6. The method of claim 1, wherein the **CpG**-S motifs in the **immunostimulatory** nucleic acid construct comprise a **CpG** motif having the formula: 5'$X_1CGX_2$3' wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine.

7. The method of claim 6, wherein the **CpG**-S motif is selected from the group consisting of GACGTT ,AGCGTT, AACGCT GTCGTT and AACGAT.

8. The method of claim 6, wherein the **CpG**-S motif comprises GTCGYT or TGACGTT.

9. The method of claim 6, wherein fie **CpG**-S motif comprises TGTCGYT.

10. The mdhod of claim 6, wherein the **CpG**-S motif comprises TCCATGTCGTTCCTGTCGTT (SEQ ID NO:1).

11. The method of claim 6, wherein the **CpG**-S motif comprises TCCTGACGTTCCTGACGTT (SEQ ID NO:2).

12. The method of claim 6, wherein the **CpG**-S motif comprises TCGTCGTTTTGTCGTTTGTCGTT (SEQ ID NO:3).

13. The method of claim 6, wherein the **CpG**-S motif comprises TCAACGTT.

14. The method of claim 1, wherein the antigen is a viral antigen.

15. The method of claim 14, wherein the viral antigen is from Hepatitis B virus (HBV).

16. The method of claim 15, wherein the viral antigen is HBV surface antigen.

17. The method of claim 1, wherein the nucleic acid construct further comprises regulatory sequences for expression of DNA in eukaryotic cells.

18. The method of claim 17, wherein the regulatory sequence is a promoter.

19. The method of claim 18, wherein the promoteris a viral promoter.

20. The method of claim 19, wherein the promoter is a CMV promoter.

21. The method of claim 18, wherein the promoter is insensitive to cytokine regulation.

22. The method of claim 18, wherein the promoter is cytokine sensitive.

23. The method of claim 18, wherein the promoter is a non-viral promoter.

24. The method of claim 18, wherein the promoter is a tissue- or cell-specific promoter.

25. The method of claim 24, wherein the cell specific promoter is operative in antigen-presenting cells.

26. The method of claim 25, wherein the promoter is a mammalian MHC I promoter.

27. A method for enhancing the iminunostimulatory effect of an antigen in a mammalian or avian subject, comprising administering to the subject an effective amount of the **immunostimulatory** nucleic acid construct of claim 1 encoding the antigen, wherein the antigen is selected from the group consisting of a mammalian antigen, an avian antigen, an antigen from a pathogen that infects mammalian and avian subjects, wherein the pathogen is selected from the group consisting of a bacterium, a virus, a fungus and a parasite.

28. The method of claim 1, wherein the antigen is a bacterial antigen.

29. The method of claim 1, wherein the antigen is derived from a parasite.

30. A method of eliciting an immune response against an antigen in a mammalian or avian subject comprising: administering to the subject an effective amount of an antigen-encoding **immunostimulatory** nucleic acid construct comprising at least one **CpG**-S motif and produced by determining CpGN and **CpG**-S motifs present in an antigen-encoding nucleic acid construct comprising at least one **CpG**-S motif; and removing **CpG**-N motifs from the nucleic acid construct and optionally inserting **CpG**-S motifs into the nucleic acid construct, thereby eliciting an immune response against the antigen in the mammalian or avian subject, wherein the **CpG**-N motifs comprise motifs selected from the group consisting of direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides, a combination thereof, and a poly-G motif, wherein the poly-G motif optionally contains at least four Gs in a row or two G trimers, wherein the **CpG**-S motifs comprise motifs having the formula $5'X_{1CGX23}'$ wherein C is unmethylated, at least one nucleotide separates consecutive CG dinucleotides in the motif X_1 is selected from the group consisting of adenine, guanine, and thymine and X_2

is selected from the group consisting of cytosine, thymine; and adenine, wherein the **CpG**-N motifs are removed from non-essential regions of the nucleic acid construct and the **CpG**-S motifs are inserted into non-essential regions of the nucleic acid construct.

31. The method of claim 30, wherein the nucleic acid construct further comprises regulatory sequences for expression of DNA in eukaryotic cells.

32. The method of claim 31, wherein the regulatory sequence is a promoter.

33. The method of claim 32, wherein the promoter is a viral promoter.

34. The method of claim 33, wherein the promoter is a CMV promoter.

35. The method of claim 32, wherein the promoter is insensitive to cytokine regulation.

36. The method of claim 32, wherein the promoter is cytokine sensitive.

37. The method of claim 32, wherein the promoter is a non-viral promoter.

38. The method of claim 32, wherein the promoter is a tissue-specific promoter.

39. The method of claim 32, wherein the promoter is a cell-specific promoter.

40. The method of claim 39, wherein the cell-specific promoter is operative in antigen-presenting cells.

41. The method of claim 40, wherein the promoter is a mammalian MHC I promoter.

42. The method of claim 30, wherein the antigen is a viral antigen.

43. The method of claim 42, wherein the viral antigen is from Hepatitis B virus (HBV).

44. The method of claim 30, wherein the **CpG**-N motifs are selected from the group consisting of clusters of direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides and a combination thereof.

45. The method of claim 30, wherein the nucleic acid construct is a plasmid.

46. The method of claim 30, wherein the nucleic acid construct is a viral vector.

47. The method of claim 30, wherein the **CpG**-S motifs in the **immunostimulatory** nucleic acid construct comprise a **CpG** motif having the formula: 5'$X_1CGX_2$3' wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine.

48. The method of claim 47, wherein the **CpG**-S motif is selected from the group consisting of GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT.

49. The method of claim 47, wherein the **CpG**-S motif comprises GTCGYT or TGACGTT.

50. The method of claim 47, wherein the **CpG**-S motif comprises TGTCGYT.

51. The method of claim 47, wherein the **CpG**-S motif comprises TCCATGTCGTTCCTGTCGTT (SEQ ID NO:1).

52. The method of claim 47, wherein the **CpG**-S motif comprises TCCTGACGTTCCTGACGTT (SEQ ID NO:2).

53. The method of claim 47, wherein the **CpG**-S motif comprises TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:3).

54. The method of claim 47, wherein the **CpG**-S motif comprises TCAACGTT.

55. The method of claim 30, wherein the antigen is derived from a parasite.

56. The method of claim 30, further comprising administering an antigen to the subject.

57. The method of claim 56, wherein the antigen is administered to the subject essentially simultaneously with the **immunostimulatory** nucleic acid construct.

58. The method of claim 30, wherein the antigen is a bacterial antigen.

59. The method of claim 30, wherein the antigen is derived from a parasite.

60. A method for producing an **immunostimulatory** nucleic acid construct comprising at least one **CpG**-S motif and a nucleic acid encoding an antigen comprising: determining **CpG**-N and **CpG**-S motifs present in a nucleic acid construct comprising at least one **CpG**-S motif; removing **CpG**-N motifs from the nucleic acid construct; and optionally inserting **CpG**-S motifs into the nucleic acid construct, then inserting the nucleic acid encoding the antigen into the nucleic acid construct, thereby producing said **immunostimulatory** nucleic acid construct that stimulates an immune response against the antigen, wherein the **CpG**-N motifs comprise motifs selected from the group consisting of direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides, a combination thereof, and a poly-G motif, wherein the poly-G motif optionally contains at least four Gs in a row or two G trimers, and wherein the **CpG**-S motifs comprise motifs having the formula $5'X_{1CGX23}'$ wherein C is unmethylated, at least one nucleotide separates consecutive CG dinucleotides in the motif, X_1 is selected from the group consisting of adenine, guanine, and thymine and X_2 is selected from me group consisting of cytosine, thymine, and adenine, and wherein the **CpG**-N motifs are removed from non-essential regions of the nucleic acid construct and the **CpG**-S motifs arc inserted into non-essential regions of the nucleic acid construct, wherein the antigen is selected from the group consisting of a mammalian antigen, an avian antigen, an antigen from a pathogen that infects mammalian and avian subjects, wherein the pathogen is selected from the group consisting of a bacteriun, a virus, a fungus and a parasite.

61. The method of claim 60, wherein the **CpG**-N motifs are selected from the group consisting of clusters of direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetmanucleotides, CGCG tetranucleotides and a combination thereof.

62. The method of claim 60, wherein the nucleic acid construct is a plasmid.

63. The method of claim 60, wherein the nucleic acid construct is a viral vector.

64. The method of claim 60, wherein tie **CpG**-S motifs in the **immunostimulatory** nucleic acid construct comprise a **CpG** motif having the formula: $5'X_{1CGX23}'$ wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine.

65. The method of claim 64, wherein the **CpG**-S motif is selected from the group consisting of GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT.

66. the method of claim 64, wherein the **CpG**-S motif comprises GTCGYT or TGACGTT.

67. The method of claim 64, wherein the **CpG**-S motif comprises TGTCGYT.

68. The method of claim 64, wherein the **CpG**-S motif comprises TCCATGTCGTTCCTGTCGTT (SEQ ID: NO:1).

69. The method of claim 64, wherein the **CpG**-S motif comprises TCCTGACGTTCCTGACGTT (SEQ ID NO:2).

70. The method of claim 64, wherein the **CpG**-S motif comprises TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:3).

71. The method of claim 64, wherein the **CpG**-S motif comprises TCAACGTT.

72. The method of claim 60, wherein the antigen is a viral antigen.

73. The method of claim 72, wherein the viral antigen is firom Hepatitis

B virus (HBV).

74. The method of claim 73, wherein the viral antigen is HBV surface antigen.

75. The method of claim 65, further comprising inserting to the nucleic acid construct regulatory sequences for expression of DNA in eukaryotic cells.

76. The method of claim 75, wherein the regulatory sequence is a promoter.

77. The method of claim 76, wherein the promoter is a viral promoter.

78. The method of claim 77, wherein the promoter is a CMV promoter.

79. The method of claim 76, wherein the promoter is a tissue- or cell-specific promoter.

80. The method of claim 79, wherein the cell specific promoter is operative in antigen-presenting cells.

81. The method of claim 80, wherein the promoter is a mammalian MHC I promoter.

82. The method of claim 60, wherein the antigen is a bacterial antigen.

83. The method of claim 60, wherein the antigen is derived from a parasite.

84. A method for enhancing the **immunostimulatory** effect of an antigen in a mammalian or avian subject, comprising administering to the subject an effective amount of the **immunostimulatory** nucleic acid construct of claim 60 encoding the antigen, wherein the antigen is selected from the group consisting of a mammalian antigen, an avian antigen, an antigen from a pathogen that infects mamalian and avian subjects, wherein the pathogen is selected from the group consisting of a bacterium, a virus, a fungus and a parasite.

85. A method of eliciting an immune response against an antigen in a mammalian or avian subject comprising: administering to the subject an effective amount of an antigen-encoding **immunostimulatory** nucleic acid construct comprising at least one **CpG**-S motif and produced by determining **CpG**-N and **CpG**-S motifs present in a nucleic acid construct comprising at least one **CpG**-S motif; and removing **CpG**-N motifs from the nucleic acid construct, optionally inserting **CpG**-S motifs into the nucleic acid construct, and then inserting a nucleic acid encoding an antigen into the nucleic acid construct, thereby eliciting an immune response against the antigen in the mammalian or avian subject, wherein the **CpG**-N motifs comprise motifs selected from the group consisting of direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides, a combination thereof, and a poly-G motif, wherein the poly-G motif optionally contains at least four Gs in a row or two G trimers, wherein the **CpG**-S motifs comprise motifs having the formula $5'X_1CGX_2 3'$ wherein C is unmethylated, at least one nucleotide separates consecutive CG dinucleotides in the motif, X_1 is selected from the group consisting of adenine, guanine, and thymine and X_2 is selected from the group consisting of cytosine, thymine, and adenine, wherein the **CpG**-N motifs are removed from non-essential regions of the nucleic acid construct and the **CpG**-S motifs are inserted into non-essential regions of the nucleic acid construct.

86. The method of claim 85, further comprising inserting into the nucleic acid construct regulatory sequences for expression of DNA in eukaryotic cells.

87. The method of claim 86, wherein the regulatory sequence is a promoter.

88. The method of claim 87, wherein the promoter is a viral promoter.

89. The method of claim 87, wherein the promoter is a CMV promoter.

90. The method of claim 87, wherein the promoter is a tissue-specific promoter.

91. The method of claim 87, wherein the promoter is a cell-specific promoter.

92. The method of claim 91, wherein the cell-specific promoter is operative in autigen-presenting cells.

93. The method of claim 92, wherein the promoter is a mammalian MHC I promoter.

94. The method of claim 85, wherein the antigen is a viral antigen.

95. The method of claim 94, wherein the viral antigen is from Hepatitis B virus (HBV).

96. The method of claim 85, wherein the **CpG**-N motifs are selected from the group consisting of clusters of direct repeats of **CpG** dinucieotides, CCG trinucleotides; CGG trinucleotides, CCGG tetranuclootides, CGCG tetranucleotides and a combination thereof.

97. The method of claim 85, wherein the nucleic acid construct is a plasmid.

98. The method of claim 85, wherein the nucleic acid construct is a viral vector.

99. The method of claim 85, wherein the **CpG**-S motifs in the itmmunostimulatory nucleic acid construct comprise a **CpG** motif having the formula: 5'X_{1CGX23}' wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine.

100. The method of claim 99, wherein the **CpG**-S motif is selected from the group consisting of GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT.

101. The method of claim 99, wherein the **CpG**-S motif comprises GTCGYT or TGACGTT.

102. The method of claim 99, wherein the **CpG**-S motif comprises TGTCGYT.

103. The method of claim 99, wherein the **CpG**-S motif comprises TCCATGTCGTTCCTGTCGTT (SEQ ID NO:1).

104. The method of claim 99, wherein the **CpG**-S motif comprises TCCTGACGTTCCTGACGTT (SEQ ID NO:2).

105. The method of claim 99, wherein the **CpG**-S motif comprises TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:3).

106. The method of claim 99, wherein the **CpG**-S motif comprises TCAACGTT.

107. The method of claim 85, wherein the antigen is a bacterial antigen.

108. The method of claim 85, wherein the antigen is derived from a parasite.

109. The method of claim 85, further comprising administering an antigen to the subject.

AI **US 1998-82649 19980520 (9)**

AB The present invention shows that DNA vaccine vectors can be improved by removal of **CpG**-N motifs and optional addition of **CpG**-S motifs. In addition, for high and long-lasting levels of expression, the optimized vector should include a promoter/enhancer that is not down-regulated by the cytokines induced by the **immunostimulatory CpG** motifs. Vectors and methods of use for immunostimulation are provided herein. The invention also provides improved gene therapy vectors by determining the **CpG**-N and **CpG**-S motifs present in the construct, removing stimulatory **CpG** (**CpG**-S) motifs and/or inserting neutralizing **CpG** (**CpG**-N) motifs, thereby producing a nucleic acid construct providing enhanced expression of the therapeutic polypeptide. Methods of use for such vectors. . .

SUMM This invention relates generally to immune responses and more particularly to vectors containing **immunostimulatory CpG** motifs and/or a reduced number of neutralizing motifs and methods of use for immunization purposes as well as vectors containing neutralizing motifs and/or a reduced number of **immunostimulatory CpG** motifs and methods of use for gene therapy protocols.

SUMM Bacterial DNA, but not vertebrate DNA, has direct **immunostimulatory** effects on peripheral blood mononuclear cells (PBMC) in vitro (Messina et aL, J. Immunol. 147: 1759-1764, 1991; Tokanuga et al.,. . . increased immunoglobulin (Ig) secretion (Krieg et al., Nature. 374:

546-549, 1995). In addition to its direct effects on B cells, **CpG** DNA also directly activates monocytes, macrophages, and dendritic cells to secrete predominantly Th 1 cytokines, including high levels of IL-12. . . al., J. Immunol. 157: 1840-1845 (1996)). These stimulatory effects have been found to be due to the presence of unmethylated **CpG** dinucleotides in a particular sequence context (**CpG**-S motifs) (Krieg et al., 1995, supra). Activation may also be triggered by addition of synthetic oligodeoxynucleotides (ODN) that contain **CpG**-S motifs (Tokunaga et al., Jpn. J Cancer Res. 79: 682-686 1988; Yi et al., J. Immunol. 156: 558-564, 1996; Davis. . .

SUMM Unmethylated **CpG** dinucleotides are present at the expected frequency in bacterial DNA but are under-represented and methylated in vertebrate DNA (Bird, Trends in Genetics. 3: 342-347, 1987). Thus, vertebrate DNA essentially does not contain **CpG** stimulatory (**CpG**-S) motifs and it appears likely that the rapid innnune activation in response to **CpG**-S DNA may have evolved as one component of the innate immune defense mechanisms that recognize structural patterns specific to microbial. .

SUMM . . . all DNA viruses and retroviruses appear to have escaped the defense mechanism of the mammalian immune system to respond to **immunostimulatory CpG** motifs. In most cases this has been accomplished through reducing their genomic content of **CpG** dinucleotides by 50-94% from that expected based on random base usage (Karlin et al., J Virol. 68: 2889-2897, 1994). **CpG** suppression is absent from bacteriophage, indicating that it is not an inevitable result of having a small genome. Statistical analysis indicates that the **CpG** suppression in lentiviruses is an evolutionary adaptation to replication in a eukaryotic host (Shaper et al., Nucl. Acids Res. 18:. . .

SUMM . . . all DNA viruses and retroviruses appear to have evolved to avoid this defense mechanism through reducing their genomic content of **CpG** dinucleotides by 50-94% from that expected based on random base usage. **CpG** suppression is absent from bacteriophage, indicating that it is not an inevitable result of having a small genome. Statistical analysis indicates that the **CpG** suppression in lentiviruses is an evolutionary adaptation to replication in a eukaryotic host. Adenoviruses, however, are an exception to this rule as they have the expected level of genomic **CpG** dinucleotides. Different groups of adenovirae can have quite different clinical characteristics. Serotype 2 and 5 adenoviruses (Subgenus C) are endemic. . .

SUMM Despite high levels of unmethylated **CpG** dinucleotides, serotype 2 adenoviral DNA surprisingly is nonstimulatory and can actually inhibit activation by bacterial DNA. The arrangement and flanking bases of the **CpG** dinucleotides are responsible for this difference. Even though type 2 adenoviral DNA contains six times the expected frequency of **CpG** dinucleotides, it has **CpG**-S motifs at only one quarter of the frequency predicted by chance. Instead, most **CpG** motifs are found in clusters of direct repeats or with a C on the 5' side or a G on the 3' side. It appears that such **CpG** motifs are immune-neutralizing (**CpG**-N) in that they block the Th1-type immune activation by **CpG**-S motifs in vitro. Likewise, when **CpG**-N ODN and **CpG**-S are administered with antigen, the antigen-specific immune response is blunted compared to that with **CpG**-S alone. When **CpG**-N ODN alone is administered in vivo with an antigen, Th2-like antigen-specific immune responses are induced.

SUMM B cell activation by **CpG**-S DNA is T cell independent and antigen non-specific. However, B cell activation by low concentrations of **CpG** DNA has strong synergy with signals delivered through the B cell antigen receptor for both B cell proliferation and Ig. . . 1995, supra). This strong synergy between the B cell signaling pathways triggered through the B cell antigen receptor and by **CpG**-S DNA promotes antigen specific immune responses. The strong direct effects (T cell independent) of **CpG**-S DNA on B cells, as well as the induction of cytokines which could have indirect effects on B-cells via T-help pathways, suggests utility of **CpG**-S DNA as a vaccine adjuvant. This could be applied either to classical antigen-based vaccines or to DNA vaccines. **CpG**-S ODN have potent Th-1 like adjuvant effects with protein antigens (Chu et al., J Exp. Med. 186: 1623-1631 1997; Lipford. . .

SUMM The present invention is based on the discovery that removal of neutralizing motifs (e.g., **CpG**-N or poly G) from a vector used for immunization purposes, results in an antigen-specific **immunostimulatory** effect greater than with the starting vector. Further, when neutralizing motifs (e.g., **CpG**-N or poly G) are removed from the vector and stimulatory **CpG**-S motifs are inserted into the vector, the vector has even more enhanced **immunostimulatory** efficacy.

SUMM In a first embodiment, the invention provides a method for enhancing the **immunostimulatory** effect of an antigen encoded by nucleic acid contained in a nucleic acid construct including determining the **CpG**-N and **CpG**-S motifs present in the construct and removing neutralizing

CpG (**CpG**-N) motifs and optionally inserting stimulatory **CpG** (**CpG**-S) motifs in the construct, thereby producing a nucleic acid construct having enhanced **immunostimulatory** efficacy. Preferably, the **CpG**-S motifs in the construct include a motif having the formula 5' X_{1CGX2} 3' wherein at least one nucleotide separates consecutive. . .

SUMM . . . subject. The method includes administering to the subject an effective amount of a nucleic acid construct produced by determining the **CpG**-N and **CpG**-S motifs present in the construct and removing neutralizing **CpG** (**CpG**-N) motifs and optionally inserting stimulatory **CpG** (**CpG**-S) motifs in the construct, thereby producing a nucleic acid construct having enhanced **immunostimulatory** efficacy and stimulating a protective or therapeutic immune response in the subject. Preferably, the nucleic acid construct contains a promoter. . .

SUMM . . . wherein the polypeptide is encoded by a nucleic acid contained in a nucleic acid construct. The method includes determining the **CpG**-N and **CpG**-S motifs present in the construct, optionally removing stimulatory **CpG** (**CpG**-S) motifs and/or inserting neutralizing **CpG** (**CpG**-N) motifs, thereby producing a nucleic acid construct providing enhanced expression of the therapeutic polypeptide.

SUMM . . . vivo. The method includes administering to a subject a nucleic acid construct, wherein the construct is produced by determining the **CpG**-N and **CpG**-S motifs present in the construct and optionally removing stimulatory **CpG** (**CpG**-S) motifs and/or inserting neutralizing **CpG** (**CpG**-N) motifs, thereby enhancing expression of the therapeutic polypeptide in the subject.

DRWD . . . 2022, 2023 and 2042). Three S-ODN (1911, 1982 and 2017) and two SOS-ODN (1972 and 2042) did not contain any **immunostimulatory CpG** motifs. One S-ODN (1628) and three SOS-ODN (1585, 1972, 1981) had poly-G ends and one SOS-ODN (2042) had a poly-G. . . The (*) indicates ODN of identical sequence but different backbone: 1826 (S-ODN), 1980 (SOS-ODN) and 2061 (O-ODN). All S-ODN (both **CpG** and non-**CpG**) resulted in decreased luciferase activity whereas SOS-ODN did not unless they had poly-G sequences.

DRWD FIG. 8: Temporal and spatial separation of **CpG** ODN and plasmid DNA. The figure shows the effect of temporal or spatial separation of plasmid DNA and S-ODN on. . . muscles 3 or 14 days after they were injected with 10 μg pCMV-luc DNA. Some animals also received 10 jg **CpG**-S ODN which was mixed with the DNA vaccine or was given at the same time but at a different site,. . .

DRWD FIG. 9: Immunization of BALB/c mice with **CpG**-optimized DNA vaccines. The figure shows the enhancement of in vivo immune effects with optimized DNA vaccines. Mice were injected with. . .

DRWD FIG. 10 shows induction of a Th2-like response by a **CpG**-N motif and inhibition of the Th1-like response induced by a **CpG**-S motif. Anti-HBs antibody titers (IgG1 and IgG2a subclasses) in BALB/c mice 12 weeks after IM immunization with recombinant HBsAg, which. . . μg of neutralizing ODN (1631, CGCGCGCGCGCGCGCGCGCG(SEQ. ID NO: 22); 1984, TCCATGCCGTTCCTGCCGTT(SEQ. ID NO: 7b); or 2010 GCGGCGGGCGGCGCGCGCCC(SEQ. ID NO: 75); **CpG** dinucleotides are underlined for clarity) or with 10 μg stimulatory ODN+10 μg neutralizing ODN. To improve nuclease resistance for these. . .

DETD The present invention provides vectors for immunization or therapeutic purposes based on the presence or absence of **CpG** dinucleotide immunomodulating motifs. For immunization purposes, inununostimulatoxy motifs (**CpG**-S) are desirable while immunoinhibitory **CpG** motifs (**CpG**-N) are undesirable, whereas for gene therapy purposes, **CpG**-N are desirable and **CpG**-S are undesirable. Plasmid DNA expression cassettes were designed using **CpG**-S and **CpG**-N motifs. In the case of DNA vaccines, removal of **CpG**-N motifs and addition of **CpG**-S motifs should allow induction of a more potent and appropriately directed immune response. The opposite approach with gene therapy vectors, namely the removal of **CpG**-S motifs and addition of **CpG**-N motifs, allows longer lasting therapeutic effects by abrogating immune responses against the expressed protein.

DETD . . . comprise antigen-expressing plasmid DNA vectors. Since such plasmids are produced in bacteria and then purified, they usually contain several unmethylated **immunostimulatory CpG**-S motifs. There is now convincing evidence that the presence of such motifs is essential for the induction of immune responses. . . Krieg et al., Trends Microbiology. 6: 23-27, 1998). For example, it has been shown that removal or methylation of potent **CpG**-S sequences from plasmid DNA vectors reduced or abolished the in vitro production of Th1 cytokines (e.g., IL-12, IFN-α, IFN-γ) from. . . (Sato et al., 1996, supra; Klimnan et al., J. Immunol 158: 3635-3639 (1997). Potent responses could be restored by cloning **CpG**-S motifs back into the vectors (Sato et al., 1996, supra) or by coadministering **CpG**-S ODN (Klinan et al., 1997, supra). The humoral response in monkeys to a DNA vaccine can also be augmented by. . .

DETD The present invention shows that DNA vaccine vectors can be improved by removal of **CpG**-N motifs and further improved by the addition of **CpG**-S motifs. In addition, for high and long-lasting levels of expression, the optimized vector should preferably include a promoter/enhancer, which is not down-regulated by the cytokines induced by the **immunostimulatory CpG** motifs.

DETD It has been shown that the presence of unmethylated **CpG** motifs in the DNA vaccines is essential for the induction of immune responses against the antigen, which is expressed only. . . al., 1996, Klinman et al., 1997, supra). As such, the DNA vaccine provides its own adjuvant in the form of **CpG** DNA. Since single-stranded but not double-stranded DNA can induce immunostimulation in vitro, the **CpG** adjuvant effect of DNA vaccines in vivo is likely due to oligonucleotides resulting from plasmid degradation by nucleases. Only a. . .

DETD The present invention provides DNA vaccine vectors further improved by removal of undesirable immunoinhibitory **CpG** motifs and addition of appropriate **CpG immunostimulatory** sequences in the appropriate number and spacing. The correct choice of **immunostimulatory CpG** motifs could allow one to preferentially augment humoral or CTL responses, or to preferentially induce certain cytokines.

DETD The exact **immunostimulatory CpG** motif(s) to be added will depend on the ultimate purpose of the vector. If it is to be used for. . .

DETD . . . for different companion and food-source animals which receive veterinary vaccination. There is a very strong correlation between certain in vitro **immunostimulatory** effects and in vivo adjuvant effect of specific **CpG** motifs. For example, the strength of the humoral response correlates very well ($r>0.9$) with the in vitro induction of TNF-α,. . .

DETD . . . to be sufficient for use in DNA vaccines (Davis et al., Human Molec. Genetics. 2: 1847-1851, 1993). The use of **CpG**-optimized DNA vaccine vectors could improve immune responses to antigen expressed for a limited duration, as with these viral promoters. When a strong viral promoter is desired, down-regulation of expression may be avoidable by choosing **CpG**-S motfis that do not induce the cytokine(s) that affect the promoter (Harms and Splitter, 1995 supra).

DETD Preferably, the **CpG**-S motifs in the construct include a motif having the formula:

DETD . . . least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine. Exemplary **CpG**-S oligonucleotide motifs include GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT. Another oligonucleotide useful in the construct contains TCAACGTT. Further exemplary oligonucleotides. . .

DETD Preferably **CpG**-N motifs contain direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides or a combination of any of these motifs. In addition, the. . .

DETD Nucleotide sequences in the nucleic acid construct can be intentionally manipulated to produce **CpG**-S sequences or to reduce the number of **CpG**-N sequences for immunization vectors. For example, site-directed mutagenesis can be utilized to produce a desired **CpG** motif. Alternatively, a particular **CpG** motif can be synthesized and inserted into the nucleic acid construct. Further, one of skill in the art can produce double-stranded **CpG** oligos that have self-complementary ends that can be ligated together to form long chains or concatemers that can be ligated into a plasmid, for example. It will be apparent that the number of **CpG** motifs or **CpG**-containing oligos that can be concatenated will depend on the length of the individual oligos and can be readily determined by. . .

DETD . . . The method includes administering to the subject an immunomostimulatory effective amount of a nucleic acid construct produced by removing neutralizing **CpG** (**CpG**-N) motifs and optionally inserting stimulatory **CpG** (**CpG**-S) motifs, thereby producing a nucleic acid construct having enhanced **immunostimulatory** efficacy and stimulating a protective immune response in the subject. The construct typically further includes regulatory sequences for expression of. . .

DETD . . . realize a desired biologic effect. For example, an effective amount of a nucleic acid construct containing at least one unmethylated **CpG** for treating a disorder could be that amount necessary to induce an immune response of sufficient magnitude to eliminate a. . . such factors as the disease or condition being treated, the particular nucleic acid being administered (e.g. the number of unmethylated **CpG** motifs (-S or -N) or their location in the nucleic acid), the size of the subject, or the severity of. . .

DETD In one embodiment, the invention provides a nucleic acid construct containing **CpG** motifs as described herein as a pharmaceutical composition useful for inducing an immune response to a bacterial, parasitic, fingal, viral. . .

DETD . . . compositions can include adjuvants or additional nucleic acid constructs that express adjuvants such as cytokines or co-stimulatory

molecules. Adjuvants include **CpG** motifs such as those described in co-pending application Ser. No. 09/030,701.

DETD An "**immunostimulatory** nucleic acid molecule" or oligonucleotide as used herein refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (i.e. "**CpG** DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates (e.g. has a mitogenic effect on, or induces or increases cytokine expression by) a vertebrate lymphocyte. An **immunostimulatory** nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules may. . .

DETD Unmethylated **immunostimulatory CpG** motifs, either within a nucleic acid construct or an oligonucleotide, directly activate lymphocytes and co-stimulate antigen-specific responses. As such, they. . .

DETD In addition, an **immunostimulatory** oligonucleotide in the nucleic acid construct of the invention can be administered prior to, along with or after administration of. . . chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF. **CpG** nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Induction of NK activity and. . .

DETD . . . gene product would not be desirable. Thus, the optimal plasmid DNA cassette for gene therapy purposes will have all possible **immunostimulatory** (**CpG**-S) motifs removed and several immunoinhibitory (**CpG**-N) motifs added in. An exemplary vector for gene therapy purposes is described in the Examples.

DETD Despite comparable levels of unmethylated **CpG** dinucleotides, DNA from serotype 12 adenovirus is immune stimulatory, but serotype 2 is nonstimulatory and can even inhibit activation by bacterial DNA. In type 12 genomes, the distribution of **CpG**-flanking bases is similar to that predicted by chance. However, in type 2 adenoviral DNA the immune stimulatory **CpG**-S motifs are outnumbered by a 15 to 30 fold excess of **CpG** dinucleotides in clusters of direct repeats or with a C on the 5' side or a G on the 3' side. Synthetic oligodeoxynucleotides containing these putative neutralizing (**CpG**-N) motifs block immune activation by **CpG**-S motifs in vitro and in vivo. Eliminating 52 of the 134 **CpG**-N motifs present in a DNA vaccine markedly enhanced its Th1-like function in vivo, which was further increased by addition of **CpG**-S motifs. Thus, depending on the **CpG** motif, prokaryotic DNA can be either immune-stimulatory or neutralizing. These results have important implications for understanding microbial pathogenesis and molecular. .

DETD . . . protein, as is the case with gene replacement strategies, induces immune responses. Nevertheless, it is likely that the presence of **CpG**-S motifs aggravates this situation. The finding that removal of **CpG**-S motifs from DNA vaccines can abolish their efficacy suggests that such a strategy may prove useful for creating gene therapy vectors where immune responses against the encoded protein are undesirable. Furthermore, the more recent discovery of **CpG**-N motifs opens up the possibility of actually abrogating unwanted immune responses through incorporating such motifs into gene delivery vectors. In particular, the Th-2 bias of **CpG**-N motifs may prevent induction of cytotoxic T-cells, which are likely the primary mechanism for destruction of transfected cells.

DETD . . . polypeptide in vivo wherein the polypeptide is contained in a nucleic acid construct. The construct is produced by removing stimulatory **CpG** (**CpG**-S) motifs and optionally inserting neutralizing **CpG** (**CpG**-N) motifs, thereby producing a nucleic acid construct providing enhanced expression of the therapeutic polypeptide. Alternatively, the invention envisions using the. . .

DETD Typical **CpG**-S motifs that are removed from the construct include a motif having the formula:

DETD . . . least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine. Exemplary **CpG**-S oligonucleotide motifs include GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT. Another oligonucleotide useful in the construct contains TCAACGTT. Further exemplary oligonucleotides. . .

DETD Preferably **CpG**-N motifs contain direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides or a combination of any of these motifs. In addition, the. . .

DETD Cloning of **CpG** Optimized Plasmid DNA Vectors

DETD . . . used as the starting material to construct a basic expression vector, which was subsequently used for construction of either a **CpG**-optimized DNA vaccine vectors or a **CpG**-optimized gene therapy vectors. DNA sequences required for gene expression in eukaryotic cells were obtained by PCR using the expression vector. . .

DETD **CpG** optimized DNA vaccine vector

DETD The **CpG**-optimized DNA vaccine vectors were made from the basic

expression vector (pUK21-A2) in several steps:

DETD Site-directed mutagenesis for removal of **CpG**-N motifs, with care being taken to maintain the integrity of the open reading frame. Where necessary, the mutated sequence was. . .

DETD Addition of suitable polylinker sequence to allow easy incorporation of **CpG**-S motifs.

DETD Addition of **CpG**-S motifs which would be chosen to enhance a particular immune response (humoral, cell-mediated, high levels of a particular cytokine etc.).

DETD . . . as the starting material for construction of an optimized DNA vaccine vector. Site-directed mutagenesis was carried out to mutate those **CpG**-N sequences that were easy to mutate. As described below, 22 point-mutations were made to change a total of 15 **CpG**-N motifs to alternative non-**CpG** sequences. For 16 of these point mutations that were in coding regions, the new sequences encoded the same amino acids. . . mutated sequences were all in the kanamycin resistance gene or immediately adjacent regions. At present, we did not mutate any **CpG**-N motifs in regions with indispensable functions such as the ColE1, BGH poly A or polylinker regions, or the promoter region. . .

DETD . . . the kanamycin resistance gene and another six point-mutations within a non-essential DNA region were designed in order to eliminate immunoinhibitory **CpG**-N sequences. At this time, mutations were not made to **CpG**-N motifs contained in regions of pUK21-A that had essential functions.

DETD (iv) Insertion of **immunostimulatory** motifs into the vector pMAS

DETD The vector is now ready for cloning **CpG**-S motifs. The exact motif which would be added to the vector would depend on its ultimate application, including the species. . .

DETD . . . ACG TTC CTG ACG TTT CCA TGA CGT TCC TGA CGT TG 3' (SEQ ID NO: 12) which contains four **CpG**-S motifs (underlined), and its complementary sequence 5' GTC CAA CGT CAG GAA CGT CAT GGA AAC GTC AGG AAC GTC ATG GA 3' (SEQ ID NO:13). This sequence is based on the **CpG**-S motifs contained in oligo #1826, which has potent stimulatory effects on murine cells in vitro and is a potent adjuvant. . . of larger DNA fragments containing different copy numbers of the stimulatory motif These DNA fragments with different numbers of mouse **CpG**-S motifs were inserted into the AvaII site of pMAS, which was first dephosphorylated with CIP to prevent self-ligation. The resulting. . . to the design of the synthetic oligonucleotide sequence allowing the cloning process to be repeated until the desired number of **CpG**-S motifs were inserted. Sixteen and 50 mouse **CpG**-S motifs were inserted into the AvaHi site of pMAS, creating pMCG-16 and pMCG-50 respectively. The DNA fragment containing 50 **CpG**-S motifs was excised from pMCG-50, and inserted into HpaI-AvaII-ScaI-DraI linker of pMCG-50, creating pMCG-100. The same procedure was followed to create pMCG-200 (Table 3). Two different sequences containing human-specific **CpG**-S motifs were cloned in different numbers into pMAS to create two series of vectors, pHCG and pHIS, following the same . .

DETD . . . TTC GTG TCG TTC TTC TGT CGT CTT TA TTC TCC TGC GTG CGT CCC TTG 3' (SEQ ID NO:14) (**CpG**-S motifs are underlined). This sequence incorporates various **CpG**-S motifs that had previously been found to have potent stimulatory effects on human cells in vitro. The vector pHCG-30, pHCG-50, pHCG-100 and pHCG-200 contain 30, 50, 100 and 200 human **CpG**-S motifs respectively (Table 3).

DETD . . . TTT TGT CGT TTT GTC GTT TCG TCG TTT TGT CGT TTT GTC GTT G 3' (SEQ ID NO: 15) (**CPG**-S motifs are underlined). This sequence is based on the **CpG**-S motifs in oligo #2006, which has potent stimulatory effects on human cells in vitro The vector pHIS-40, pHIS-64, pHIS-1 28 and pHIS-1 92 contain 40, 64, 128 and 192 human **CpG** motifs respectively (Table 3).

DETD **CpG** optimized gene therapy vector

DETD (i) Site-directed mutagenesis for removal of **CpG immunostimulatory** sequences within pUK21-A2

DETD . . . following the same strategy as described previously in (ii) Site-directed mutagenesis to remove immunoinhibitoiy sequences. The point mutations eliminated 64 **CpG** stimulatory motifs resulting in the vector pGT (Table 5).

DETD Human **CpG**-N motifs were cloned into the pGTU following the same strategies as described previously in (iv) Insertion of **immunostimulatory** motifs into the vector pMAS. The oligonucleotide 5' GCC CTG GCG GGG ATA AGG CGG CGA TTT CGC GGG GGA. . . GGC CCC CGC CTT ATC CCC GCC AAA TCC CCG CCT TAT CCC CGC CAG 3' (SEQ ID NO:19) (four **CpG** motifs are underlined) were synthesized and phosphorylated. Annealing of these two oligonucleotides created a double-stranded DNA fragment, which was self-ligated. . . vector pGTU. The recombinant plasmids will be screened by restriction enzyme digestion and the vectors with the desired number of **CpG** inhibitory motifs will be sequenced and tested.

DETD In some cases, 10 or 100 μg of **CpG** ODN was added to the DNA vaccine

(pCMV-S, Davis et al., 1993b). The sequences and backbones of the ODN used. . .

DETD 1. In vitro Effects of **CpG**-N Motifs

DETD Nearly all DNA viruses and retroviruses have 50-94% fewer **CpG** dinucleotides than would be expected based on random base usage. This would appear to be an evolutionary adaptation to avoid the vertebrate defense mechanisms related to recognition of **CpG**-S motifs. **CpG** suppression is absent from bacteriophage, indicating that it is not an inevitable result of having a small genome. Statistical analysis indicates that the **CpG** suppression in lentiviruses is an evolutionary adaptation to replication in a eukaryotic host. Adenoviruses, however, are an exception to this rule as they have the expected level of genornic **CpG** dinucleotides. Different groups of adenovirae can have quite different clinical characteristics.

DETD Unlike the genome of almost all DNA viruses and retroviruses, some adenoviral genomes do not show suppression of **CpG** dinucleotides (Karlin et al., 1994; Sun et al., 1997). Analysis of different adenoviral genomes (types 2, 5, 12, and 40) reveals surprising variability among each other and compared to human and E. coli in the flanking bases around **CpG** dinucleotides (Table 7).

DETD The bases flanking **CpG** motifs determine whether a **CpG** dinucleotide will cause immune stimulation, and may also determine the type of cytokines secreted. The fact that type 2 and 5 adenoviral DNA was not only nonstimulatory but actually inhibitory of **CpG** DNA, suggested that certain nonstimulatory **CpG** motifs may even be able to block the stimulatory motifs and that the inhibitory motifs should be over-represented in the. . . human DNA). By analysis of these genomes, it was possible to identify sequences that could block the effects of known **CpG**-S sequences on in vitro B cell proliferation (Table 10) and cytokine secretion (Table 11).

DETD 2. **CpG**-S ODN cannot be used as an Adjuvant for DNA Vaccines

DETD It has previously been shown that **CpG**-S ODN is a potent vaccine adjuvant when given with HBsAg protein (Davis et al., 1998). Antibodies against HBsAg (anti-HBs) were. . . a greater proportion of IgG2a than IgG1 isotypes of antibodies in immunized BALB/c mice. The strong TH1 effect of the **CpG**-S motifs was further demonstrated by the greatly enhanced cytotoxic T-cell activity. One of the most potent **CpG**-S ODN in mice was 1826, a 20-mer with 2 **CpG**-dinucleotides and made with a synthetic phosphorothioate backbone (see Table 6 for sequence).

DETD . . . the success with protein antigens, attempts to augment immune responses induced by a HBsAg-expressing DNA vaccine by the addition of **CpG**-S ODN 1826 failed. Surprisingly, the immune responses decreased with the addition of **CpG**-S ODN in a dose-dependent manner (FIG. 6, top panel). Addition of ODN #1826 to a luciferase reporter gene construct. . . resulted in a dose-dependent decrease in luciferase expression (FIG. 6, bottom panel). This indicates that the negative effects of the **CpG**-S ODN on the DNA vaccine were due to reduced gene expression rather than an effect on the immune response against. . .

DETD . . . are present) (FIG. 7), this is not useful since SOS-ODN are apparently also not sufficiently nuclease-resistant to exert a strong **CpG** adjuvant effect (Table 12). Administering the **CpG** S-ODN at a different time or site than the plasmid DNA does not interfere with gene expression either (FIG. 8), however nor do these approaches augment responses to DNA vaccines by administering the **CpG** S-ODN at a different time or site than the plasmid DNA (Table 12). Thus it appears that the immune system must see the antigen and the **CpG**-S motif at the same time and the same place to augment antigen-specific responses. Thus, at least for the present, it appears necessary to clone **CpG** motifs into DNA vaccine vectors in order to take advantage of their adjuvant effect.

DETD **CpG**-optimized DNA Vaccines

DETD Eliminating 52 of 134 **CpG**-N motifs from a DNA vaccine markedly enhanced its ml -like function in vivo and immune responses were furter augmented by the addition of **CpG**-S motifs to the DNA vaccine vectors (FIG. 9).

DETD Titers of antibodies were increased by the removal of **CpG**-N motifs. With the addition of 16 or 50 **CpG**-S motifs, humoral responses became increasingly more Thl, with an ever greater proportion of IgG2a antibodies. The anti-BBs titer was higher with 16 than 50 **CpG**-S motifs, perhaps because the strong cytokine response with the greater number of motifs inhibited antigen expression that was driven by. . .

DETD CTL responses were likewise improved by removal of **CPG**-N motifs, and then more so by the addition of **CpG**-S motifs to the DNA vaccines.

DETD **CpG**-Optiniized Gene Therapy Vectors

DETD . . . . the kanamycin resistance gene (Wu and Davis, unpublished). To avoid disrupting the plasmid origin of replication, mutagenesis designed to eliminate **CpG**-N motifs was restricted to the kanamycin resistance gene and non-essential DNA sequences following the gene. A total of 22 point mutations were introduced to alter 15 **CpG**-N motifs (a "motif"

refers to a hexamer containing one or more **CpG** dinucleotides) containing 19 **CpG** dinucleotides, 12 of which were eliminated and 7 of which were transformed into **CpG**-S motifs. Site-directed mutagenesis was performed by overlap extension PCR as described by Ge et aL (Prosch, S., et al., Biol.. . .

DETD Another 37 **CpG**-N motifs were removed by replacing the f1 origin with a multiple cloning site. Oligonucleotides 5' GCCCTATTTTAAATTCGAAAGTACTGGAC CTGTTAACA 3' (SEQ ID NO:20). . .

DETD Next, different numbers of **CpG**-S motifs were inserted into the vector by allowing self-ligation of a 20bp DNA fragment with the sequence 5' GACTCCATGAMTTCCTGAMTTTCCATGACTTCCTGACUTTG 3'. . . site of pMAS. Recombinant clones were screened and the two vectors were chosen for further testing with 16 and 50 **CpG**-S motifs, and named pMCG16 and pMCG50 respectively.

DETD . . . from types 2 and 5 adenovirus failed to induce cytokine production (Table 8). In fact, despite their similar frequency of **CpG** dinucleotides, type 2 or 5 adenoviral DNA severely reduced the cytokine expression induced by co-administered **immunostimulatory** E. coli genomic DNA (Table 9). This indicates that type 2 and 5 adenoviral DNA does not simply lack **CpG**-S motifs, but contains sequences that actively suppress those in E. coli DNA.

DETD Identification of putative immune neutralizing **CpG**-N motifs in type 2 and 5 adenoviral genomes. To identify possible non-random skewing of the bases flanking the **CpG** dinucleotides in the various adenoviral genomes, we examined their frequency of all 4096 hexamers. The six most common hexamers in. . . frequency in the Type 12 and E. coli genomes. Remarkably, all of these over-represented hexamers contain either direct repeats of **CpG** dinucleotides, or CpGs that are preceded by a C and/or followed by a G. These **CpG**-N motifs are approximately three to six fold more common in the inunune inhibitory type 2 and 5 adenoviral genomes than. . . E. coli or non-stimulatory human genomic DNAs (Table 7). This hexamer analysis further revealed that the frequency of hexarners containing **CpG**-S motifs (e.g., GACGTT or AACGTT) in the type 2 adenoviral genome is as low as that in the human genome:. . .

DETD Effect of **CpG**-N motifs on the immune stimulatory effects of **CpG**-S motifs. To determine whether these over-represented **CpG**-N motifs could explain the neutralizing properties of type 2 and 5 adenoviral DNA, we tested the in vitro immune effects of synthetic oligodeoxynucleotides bearing a **CpG**-S motif, one or more **CpG**-N motifs, or combinations of both. An ODN containing a single **CpG**-S motif induces spleen cell production of IL-6, IL-12, and IFN-γ (ODN 1619, Table 13). However, when the 3' end of this ODN was modified by substituting either repeating **CpG** dinucleotides or a **CpG** dinucleotide preceded by a C, the level of cytokine production was reduced by approximately 50% (ODN 1952 and 1953, Table 13). ODN consisting exclusively of these neutralizing **CpG** (**CpG**-N) motifs induced little or no cytokine production (Table 14). Indeed, addition of ODN containing one or more **CpG**-N motifs to spleen cells along with the **CpG**-S ODN 1619 caused a substantial decrease in the induction of IL-12 expression indicating that the neutralizing effects can be exerted. . .

DETD To determine whether the in vivo immune activation by ODN containing **CpG**-S motifs would be reversed by **CpG**-N motifs, we immunized mice with recombinant hepatitis B surface antigen (IBsAg), with or without nuclease resistant phosphorothioate-modified ODN containing various types of **CpG** motifs. As expected, a **CpG**-S ODN promoted a high titer of antibodies against HBsAg (anti-HBs antibodies) which were predominantly of the IgG2a subclass, indicating a ThI-type immune response (FIG. 10; ODN 1826). The various **CpG**-N ODN induced either little or no production of anti-HBs antibodies (ODN 1631, 1984, and 2010) (FIG. 10). Mice immunized with combinations of **CpG**-S and **CpG**-N ODN had a reduced level of anti-HBs antibodies compared to mice immunized with **CpG**-S ODN alone, but these were still predominantly IgG2a (FIG. 10).

DETD . . . J. J., et al., Ann. Rev. Immunol., 15, 617-648 (1997)). Based on the in vivo and in vitro effects of **CpG**-N motifs, we hypothesized that their presence within a DNA vaccine would decrease its **immunostimulatory** effects. The starting vector, pUK21-A2, contained 254 **CpG** dinucleotides, of which 134 were within **CpG**-N motifs. In order to test the hypothesis that these **CpG**-N motifs adversely affected the efficacy of this vector for DNA-based vaccination, the number of **CpG**-N motifs was reduced, either by mutation or deletion. Since mutations in the plasmid origin of replication interfere with replication of. . . restricted our initial mutations to the kanainycin resistance gene and a nonessential flanking region. We were able to eliminate 19 **CpG** dinucleotides contained within 15 of the 20 **CpG**-N motifs in these regions without changing the protein sequence. The F1 origin of replication containing 37 **CpG**-N motifs and only 17 other **CpG** dinucleotides was then deleted, creating the vector pMAS. This vector was further modified by the introduction of 16 or 50 **CpG**-S

motifs, yielding vectors pMCG16 and pMCG50 respectively. The S gene for HBsAg was then cloned into these vectors downstream from. . .

DETD . . . as a DNA vaccine, the anti-HBs response at 4 and 6 weeks was substantially stronger with DNA vaccines from which **CpG**-N motifs had been deleted, and even more so when 16 **CpG**-S motifs had been inserted. The vector with 50 **CpG**-S motifs, however, was less effective at inducing antibody production than that with 16 motifs. (FIG. 11, panel A). Removal of **CpG**-N motifs and addition of **CpG**-S motifs resulted in a more than three-fold increase in the proportion of IgG2a relative to IgG1 anti-HBs antibodies, indicating an. . . This accentuated TH1 response also was demonstrated by the striking progressive increases in CTL responses induced by vectors from which **CpG**-N motifs were deleted and/or **CpG**-S motifs added (FIG. 11, panel B).

DETD The discovery of immune activating **CpG**-S motifs in bacterial DNA has led to the realization that aside from encoding genetic information, DNA can also function as. . . present results demonstrate that genomic DNA from type 12 adenovirus is immune stimulatory, compatible with its relatively high content of **CpG**-S motifs. In contrast, genomic DNA from type 2 and 5 adenoviruses is not stimulatory, but rather is immune neutralizing and blocks the cytokine induction of bacterial DNA (Tables 8 and 9). To identify possible differences in the **CpG** motifs present in these different adenoviral genomes, analyzed the genomic frequency of all hexamer sequences was analyzed. This analysis demonstrated that only the type 2 and 5 adenoviral genomes had a dramatic overrepresentation of **CpG** motifs containing direct repeats of **CpG** dinucleotides and/or CpGs preceded by a C and/or followed by a G (Table 7). Synthetic ODN containing such putative immune neutralizing (**CpG**-N) motifs not only did not induce cytokine production in vitro, but also inhibited the ability of an immune stimulatory **CpG**-S motif to induce cytokine expression (Tables 13, 14). These studies reveal that there are immune neutralizing **CpG**-N as well as stimulatory **CpG**-S motifs and that there is a surprisingly complex role for the bases flanking **CpG** dinucleotides in determining these immune effects. In general, **CpG**-N motifs oppose **CpG**-S motifs in cis or trans. The mechanism through which **CpG**-N motifs work is not yet clear, but does not appear to involve competition for cell uptake or binding to a **CpG**-S-specific binding protein. Further studies are underway to determine the molecular mechanisms through which **CpG**-N and **CpG**-S motifs exert their respective immune effects.

DETD The hexamers that contain **CpG**-N motifs are from 15 to 30 times more common in type 2 and 5 adenoviral genomes than those that contain immune stimulatory **CpG**-S motifs. However, in type 12 adenoviral genomes the frequencies of hexamers containing **CpG**-N and **CpG**-S motifs do not differ substantially from chance. These data suggest that the immune neutralizing effects of types 2 and 5. . . are not merely a result of their propagation in eukaryotic cells, but rather are due to the overall excess of **CpG**-N compared to **CpG**-S motifs. It is tempting to speculate that the marked over-representation of **CpG**-N motifs in the genomes of types 2 and 5 adenovirus may contribute to the biologic properties, such as persistent infection of lymphocytes, which distinguish them from type 12 adenovirus. The presence of large numbers of **CpG**-N motifs within these adenoviral genomes may have played an important role in the evolution of this virus by enabling it to avoid triggering **CpG**-induced immune defenses. It will be interesting to determine the general distribution of **CpG**-N and **CpG**-S motifs in different families of microbial and viral genomes, and to explore their possible roles in disease pathogenesis.

DETD **CpG**-N motifs are also over-represented in the human genome, where their hexamers are approximately two to five-fold more common than **CPG**-S motifs. While this skewing is far less marked than that in adenoviral DNA, it would still be expected to reduce or eliminate any immune stimulatory effect from the unmethylated CpGs present in **CpG** islands within vertebrate DNA. We and others have found that even when predominantly or completely unmethylated, vertebrate DNA is still. . . P. Jones, unpublished data) (Sun, S., et al., J. Immunol., 159:3119-3125 (1997)) which is in keeping with its predominance of **CpG**-N motifs (Table 7). Given the overall level of **CpG** suppression in the human genome, the molecular mechanisms responsible for the skewing of the frequency of **CpG**-N to **CpG**-S motifs are unclear. Such a distortion from the expected random patterns would seem to require the existence of pathways that preferentially mutate the flanking bases of **CPG**-S motifs in vertebrate genomes, but do not affect **CPG**-N motifs. Indeed, statistical analyses of vertebrate genomes have provided evidence that CpGs flanked by A or T (as in **CpG**-S motifs) mutate at a faster rate than CpGs flanked by C or G (Bains, W., et al., Mutation Res., 267:43-54. . .

DETD Based on our in vitro experiments we hypothesized that the presence of **CpG**-N motifs in DNA vaccines interferes with the induction of the desired immune response. Indeed, the present study demonstrates that

elimination of **CpG**-N motifs from a DNA vaccine leads to improved induction of antibodies. By removing 52 of the **CPG**-N motifs from a DNA vaccine (45 were deleted and 7 turned into **CpG**-S motifs) the serologic response was more than doubled; by then adding an additional 16 **CpG**-S motifs, the response was enhanced nearly 10 fold (FIG. 11, panel A). Likewise, CTL responses were improved by removing **CpG**-N motifs and even more so by adding 16 or 50 **CpG**-S motifs (FIG. 11, panel B). These increased responses are especially notable in view of the fact that the total number of **CpG** dinucleotides in the mutated vaccines is considerably below the original number.

DETD The finding that the vector with 50 **CpG**-S motifs was inferior to that with 16 motifs for induction of humoral immunity was unexpected, and may be secondary to **CpG**-induced production of type I interferons, and subsequent reduction in the amount of antigen expressed. The decreased antibody response induced by. . . Our present results indicate that attaining the full clinical potential of DNA vaccines will require using engineered vectors in which **CpG**-N motifs have been deleted, and **CpG**-S motifs added.

DETD On the other hand, the field of gene therapy may benefit from the discovery of **CpG**-N motifs through their insertion into gene transfer vectors to prevent or reduce the induction of host immune responses. Most of the **CpG**-N motifs in the adenoviral genome are in the left hand (5') side, which is generally partially or totally deleted for. . . the "gutless" vectors (Kochanek, S., et al., Proc. Natl. Acad. Sci. USA, 93:5731-5736 (1996)). This could lead to an enhanced **CpG**-S effect. Since nucleic acids produced in viral vectors are unmethylated, they may produce inflammatory effects if they contain a relative excess of **CpG**-S over **CpG**-N motifs and are delivered at an effective concentration (about 1 μg/ml). Gene therapy studies with adenoviral vectors have used doses. . . are noninfectious, this corresponds to a DNA dose of approximately 40 μg/ml, which is well within the range at which **CpG** DNA causes in vivo immune stimulatory effects; just 10 gg/mouse induces IFN-γ production acts as an adjuvant for immunization (D)avis,. . . when delivered into mouse airways (Schwartz, D., et al., J. Clin. Invest., 100:68-73 (1997)). Multiple mechanisms besides the presence of **CpG**-S DNA are doubtless responsible for the inflammatory responses that have limited the therapeutic development of adenoviral vectors (Newman, K. D.,. . . J. Clin. Invest., 97:1504-1511 (1996)). Nonetheless, our present results suggest that consideration be given to the maintenance or insertion of **CpG**-N motifs in adenoviral vectors, and to the engineering of backbones and inserts so that **CpG**-S motifs are mutated in order to reduce immune activation.

DETD . . . safety of repeatedly delivering high doses of DNA. Since the plasmids used for gene therapy typically contain several hundred unmethylated **CpG** dinucleotides, many of which are in **CpG**-S motifs, some immune activation may be expected to occur. Indeed, mice given repeated doses of just 10 μg of plasmid. . . onset of symptoms compatible with immune activation, including fever, chills, and pulmonary congestion. Another reason to avoid the presence of **CpG**-S motifs in gene therapy vectors is that the cytokines that are produced due to the immune stimulation may reduce plasmid. . .

DETD . . . highly desirable to develop improved gene delivery systems with reduced immune activation. It is not possible to simply methylate tie **CpG**-S dinucleotides in gene therapy plasmids, since methylation of promoters abolishes or severely reduces their activity. The only promoter resistant to. . . greatly reduced if the coding sequences are methylated. In fact, even the strong CMV IE promoter is completely inactivated by **CpG** methylation. Deletion of all CpGs from an expression plasmid is not feasible since many of these are located in the. . . Kb long) where even single base changes can dramatically reduce plasmid replication. For these reasons, we propose that addition of **CpG**-N motifs, and/or mutation or conversion of **CpG**-S to **CpG**-N motifs may lead to the generation of less immune stimulatory vectors for gene therapy. Studies to investigate this possibility are. . .

DETD

TABLE 3

Plasmids containing **immunostimulatory CpG** motifs

Plasmid	Backbone	No. **CpG**, Motifs	Species Specificity and ODN Equivalence of **CpG**-S Insert
pMCG-16	pMAS	16	mouse-specific **CpG** motif
pMCG-50	pMAS	50	#1826[1]
pMCG-100	pMAS	100	
pMCG-200	pMAS	200	
pHCG-30	pMAS	30	human-specific **CpG** motif -
pHCG-50	pMAS	50	no ODN equivalent[2]

pHCG-100 pMAS 100
pHCG-200 pMAS 200
pHIS-40 pMAS 40 human-specific **CpG** motif
pHIS-64 pMAS 64 #2006[3]
pHIS-128 pMAS 128
pHIS-192 pMAS 192

[1] sequence of 1826 is **TCCATGACGTTCCTGACGTT**
[2] sequence used as a source of **CpG** motifs is
GACTTCGTGTCGTTCTTCTGTCGTCTTTAGCGCTTCTCCTGCGTGCGTCCCTTG(SEQ ID NO:14)
[3] sequence of 2006 is TCGTCGTTTTGTCGTTTTGTCGTT(SEQ ID NO:3)
DETD
TABLE 6

ODN used with plasmid DNA
ODN
Backbone code number Sequence

(SEQ ID NO:51)
S-ODN 1826 **TCCATGACGTTCCTGACGTT**

(SEQ ID NO:52)
1628 GGGGTCAACGTTGAGGGGGG

(SEQ ID NO:53)
1911 TCCAGGACTTTCCTCAGGTT

(SEQ ID NO:54)
1982 TCCAGGACTTCTCTCAGGTT

(SEQ ID NO:55)
2017 CCCCCCCCCCCCCCCCCCCC

(SEQ ID NO:56)
O-ODN 2061 **TCCATGACGTTCCTGACGTT**

(SEQ ID NO:57)
2001 GGCGGCGGCGGCGGCGGCGG

(SEQ ID NO:58)
SOS-ODN 1980 **TCCATGACGTTCCTGACGTT**

(SEQ ID NO:59)
1585 GGGTCAACGTTGAGGGGGG

(SEQ ID NO:60)
1844 TCTCCCAGCGTGCGCCATAT

(SEQ ID NO:61)
1972 GGGGTCTGTGCTTTTGGGGGG

(SEQ. . .
DETD . . . 0.388
CGCGCG 1.336 0.322 0.379 0.106
GCCGCC 1.280 0.410 0.466 0.377
CGCCGC 1.252 0.410 0.623 0.274
GACGTT 0.083 0.234 0.263 0.068
AACGTT 0.056 0.205 0.347 0.056
(**CpG**-S)
DETD . . . 5 adenovirus are essentially identical to those in type 2, and are therefore not shown. The last two hexamers are **CpG**-S motifs shown for comparison and are the most stimulatory of all tested **CpG**-S motifs.
DETD
TABLE 10

Inhibitory **CpG** motifs can block B cell proliferation induced by a stimulatory **CpG** motif
Oligonucleotide added cpm

medium 194
1668 (TCCATGACGTTCCTGATGCT)(SEQ ID NO:68) 34,669
1668 + 1735 (GCGTTTTTTTTTGCG)(SEQ ID NO:69) 24,452
1720 (TCCATGAGCTTCCTGATGCT)(SEQ ID NO:70) 601
1720 + 1735. . .
DETD . . . 48 hr. The cells were then pulsed with ^{3H} thymidine, harvested, and the cpm determined by scintillation counting. The stimulatory **CpG** oligo 1668 was slightly but significantly inhibited by the inhibitory motifs in oligo 1735. The non **CpG** oligo 1720 is

included as a negative control. (SEQ ID NO:68-70, respectively).
DETD
TABLE 11

Inhibitory effects of "bad" **CpG** motifs
on the "good" **CpG** Oligo 1619
Oligonucleotide added IL-12 in pg/ml

medium 0
1619 alone 6
1619 + 1949 (TCCATGTCGTTCCTGATGCG(SEQ ID NO:72)) 16
1619 + 1952 (TCCATGTCGTTCCGCGCGCG(SEQ ID NO:73)). . .
DETD . . . the culture and tested for IL-12 by ELISA. All wells except the control (medium) contained 60 µg/ml of the stimulatory **CpG** oligodeoxynucleotide 1619; stimulatory (1949) and inhibitory (all other sequences have a strong inhibitory motif) oligos were added to the indicated. . .
DETD
TABLE 12

Effect of **CpG**-S ODN adjuvant on anti-HBs response in mice
immunized with HBsAg-expressing DNA vaccine (pCMV-S): comparison
of mixed formulation with temporal or spatial separation of
plasmid DNA and ODN
Site and Time Relative
CpG ODN (100 µg) DNA vaccine Anti-HBs Titer
Sequence Backbone (pCMV-S, 10 µg) at 12 wk

None -- -- 6379 ± 2126
18260 O-ODN. . .
DETD
TABLE 13

Identification of neutralizing **CpG** motifs which reduce the induction of
cytokine secretion by a **CpG**-S motif in the same ODN (cis-neutralization)
ODN-induced cytokine expression[2]
ODN sequence 5'-3'[1] IL-6[2] IL-12 IFN-γ

None <5 206 898
1619 TCCATGTCGTTCCTGATGCT (SEQ ID. . . ..CC... (SEQ ID NO:74)
557 1854 2000

[1]Dots in the sequence of ODN 1952 and 1953 indicate identity to ODN 1619; **CpG** dinucleotides are underlined for clarity. ODN without **CpG**-N or **CpG**-S motifs had little or no effect on cytokine production. The data shown are representative of 4 experiments.
[2]All cytokines are. .
DETD
TABLE 14

Inhibition of **CpG**-induced cytokine secretion by ODN containing **CpG**-N motifs
ODN sequence 5'-3' IL-12 secretion[1] **CpG**-S-induced IL-12 secretion[2]

none 268 5453
1895 GCGCGCGCGCGCGCGCGCGC(SEQ ID NO:76) 123 2719
1896 CCGGCCGGCCGGCCGGCCGG(SEQ ID NO:77) 292 2740
1955 GCGGCGGGCGGCGCGCGCCC(SEQ ID NO:75) 270 2539
2037 TCCATGCCGTTCCTGCCGTT(SEQ. . .
[2]Cells were set up the same as in [1] except that IL-12 secretion was induced by the addition of the **CpG** ODN 1619 (TCCATGTCGTTCCTGATGCT) at 30 µg/ml. The data shown are representative of 5 experiments.
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1. A method for producing an **immunostimulatory** nucleic acid construct comprising at least one **CpG**-S motif and a nucleic acid encoding an antigen comprising: determining **CpG**-N and **CpG**-S motifs present in a nucleic acid construct comprising at least one **CpG**-S motif and a nucleic acid encoding an antigen; removing **CpG**-N motifs from the nucleic acid construct; and optionally inserting **CpG**-S motifs into the nucleic acid construct, thereby producing said **immunostimulatory** nucleic acid construct that stimulates an immune response against the antigen, wherein the **CpG**-N motifs comprise motifs selected from the group consisting of direct repeats of **CpG** dinucleotides, CCG trinuclectides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides, a combination thereof, and a poly-G motif, wherein the poly-G motif optionally contains at least four Gs in a row or two G trimers, and wherein the **CpG**-S motifs comprise motifs having the formula 5'X_{1CGX23}' wherein C is unmethylated, at least one nucleotide separates consecutive CG dinucleotides in. . . adenine, guanine, and thymine and X_2 is selected from the group consisting of cytosine, thymine, and adenine, and wherein the **CpG**-N motifs are removed from non-essential regions of the nucleic acid construct and the **CpG**-S motifs are inserted into non-essential regions of the nucleic acid construct, wherein the antigen is selected from the group consisting. . .

2. The method of claim 1, wherein the **CpG**-N motifs are removed by site-specific mutagenesis.

3. The method of claim 1, wherein the **CpG**-N motifs are selected from the group consisting of clusters of direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucilcotides, CGCG tetranucleotides and a combination thereof.

6. The method of claim 1, wherein the **CpG**-S motifs in the **immunostimulatory** nucleic acid construct comprise a **CpG** motif having the formula: 5'$X_1CGX_2$3' wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and.

7. The method of claim 6, wherein the **CpG**-S motif is selected from the group consisting of GACGTT ,AGCGTT, AACGCT GTCGTT and AACGAT.

8. The method of claim 6, wherein the **CpG**-S motif comprises GTCGYT or TGACGTT.

9. The method of claim 6, wherein fie **CpG**-S motif comprises TGTCGYT.

10. The mdhod of claim 6, wherein the **CpG**-S motif comprises TCCATGTCGTTCCTGTCGTT (SEQ ID NO:1).

11. The method of claim 6, wherein the **CpG**-S motif comprises TCCTGACGTTCCTGACGTT (SEQ ID NO:2).

12. The method of claim 6, wherein the **CpG**-S motif comprises TCGTCGTTTTGTCGTTTGTCGTT (SEQ ID NO:3).

13. The method of claim 6, wherein the **CpG**-S motif comprises TCAACGTT.

. effect of an antigen in a mammalian or avian subject, comprising administering to the subject an effective amount of the **immunostimulatory** nucleic acid construct of claim 1 encoding the antigen, wherein the antigen is selected from the group consisting of a.

. against an antigen in a mammalian or avian subject comprising: administering to the subject an effective amount of an antigen-encoding **immunostimulatory** nucleic acid construct comprising at least one **CpG**-S motif and produced by determining CpGN and **CpG**-S motifs present in an antigen-encoding nucleic acid construct comprising at least one **CpG**-S motif; and removing **CpG**-N motifs from the nucleic acid construct and optionally inserting **CpG**-S motifs into the nucleic acid construct, thereby eliciting an immune response against the antigen in the mammalian or avian subject, wherein the **CpG**-N motifs comprise motifs selected from the group consisting of direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides, a combination thereof, and a poly-G motif, wherein the poly-G motif optionally contains at least four Gs in a row or two G trimers, wherein the **CpG**-S motifs comprise motifs having the formula 5'$X_1CGX_2$3' wherein C is unmethylated, at least one nucleotide separates consecutive CG dinucleotides in. . . of adenine, guanine, and thymine and X_2 is selected from the group consisting of cytosine, thymine; and adenine, wherein the **CpG**-N motifs are removed from non-essential regions of the nucleic acid construct and the **CpG**-S motifs are inserted into non-essential regions of the nucleic acid construct.

44. The method of claim 30, wherein the **CpG**-N motifs are selected from the group consisting of clusters of direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides and a combination thereof.

47. The method of claim 30, wherein the **CpG**-S motifs in the **immunostimulatory** nucleic acid construct comprise a **CpG** motif having the formula: 5'$X_1CGX_2$3' wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and.

48. The method of claim 47, wherein the **CpG**-S motif is selected from the group consisting of GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT.

49. The method of claim 47, wherein the **CpG**-S motif comprises GTCGYT or TGACGTT.

50. The method of claim 47, wherein the **CpG**-S motif comprises TGTCGYT.

51. The method of claim 47, wherein the **CpG**-S motif comprises TCCATGTCGTTCCTGTCGTT (SEQ ID NO:1).

52. The method of claim 47, wherein the **CpG**-S motif comprises TCCTGACGTTCCTGACGTT (SEQ ID NO:2).

53. The method of claim 47, wherein the **CpG**-S motif comprises TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:3).

54. The method of claim 47, wherein the **CpG**-S motif comprises TCAACGTT.

57. The method of claim 56, wherein the antigen is administered to the subject essentially simultaneously with the **immunostimulatory** nucleic acid construct.

60. A method for producing an **immunostimulatory** nucleic acid construct comprising at least one **CpG**-S motif and a nucleic acid encoding an antigen comprising: determining **CpG**-N and **CpG**-S motifs present in a nucleic acid construct comprising at least one **CpG**-S motif; removing **CpG**-N motifs from the nucleic acid construct; and optionally inserting **CpG**-S motifs into the nucleic acid construct, then inserting the nucleic acid encoding the antigen into the nucleic acid construct, thereby producing said **immunostimulatory** nucleic acid construct that stimulates an immune response against the antigen, wherein the **CpG**-N motifs comprise motifs selected from the group consisting of direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides, a combination thereof, and a poly-G motif, wherein the poly-G motif optionally contains at least four Gs in a row or two G trimers, and wherein the **CpG**-S motifs comprise motifs having the formula 5'X_{1CGX23}' wherein C is unmethylated, at least one nucleotide separates consecutive CG dinucleotides in. . . adenine, guanine, and thymine and X_2 is selected from me group consisting of cytosine, thymine, and adenine, and wherein the **CpG**-N motifs are removed from non-essential regions of the nucleic acid construct and the **CpG**-S motifs arc inserted into non-essential regions of the nucleic acid construct, wherein the antigen is selected from the group consisting. . .

61. The method of claim 60, wherein the **CpG**-N motifs are selected from the group consisting of clusters of direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetmanucleotides, CGCG tetranucleotides and a combination thereof.

64. The method of claim 60, wherein tie **CpG**-S motifs in the **immunostimulatory** nucleic acid construct comprise a **CpG** motif having the formula: 5'X_{1CGX23}' wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and. . .

65. The method of claim 64, wherein the **CpG**-S motif is selected from the group consisting of GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT.

66. the method of claim 64, wherein the **CpG**-S motif comprises GTCGYT or TGACGTT.

67. The method of claim 64, wherein the **CpG**-S motif comprises TGTCGYT.

68. The method of claim 64, wherein the **CpG**-S motif comprises TCCATGTCGTTCCTGTCGTT (SEQ ID: NO:1).

69. The method of claim 64, wherein the **CpG**-S motif comprises TCCTGACGTTCCTGACGTT (SEQ ID NO:2).

70. The method of claim 64, wherein the **CpG**-S motif comprises TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:3).

71. The method of claim 64, wherein the **CpG**-S motif comprises TCAACGTT.

84. A method for enhancing the **immunostimulatory** effect of an antigen in a mammalian or avian subject, comprising administering to the subject an effective amount of the **immunostimulatory** nucleic acid construct of claim 60 encoding the antigen, wherein the antigen is selected from the group consisting of a. . . . against an antigen in a mammalian or avian subject comprising: administering to the subject an effective amount of an antigen-encoding **immunostimulatory** nucleic acid construct comprising at least one **CpG**-S motif and produced by determining **CpG**-N and **CpG**-S motifs present in a nucleic acid construct comprising at least one **CpG**-S motif; and removing **CpG**-N motifs from the nucleic acid construct, optionally inserting **CpG**-S motifs into the nucleic acid construct, and then inserting a nucleic acid encoding an antigen into the nucleic acid construct, thereby eliciting an immune response against the antigen in the mammalian or avian subject, wherein the **CpG**-N motifs comprise motifs selected from the group consisting of direct repeats of **CpG** dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides, a combination thereof, and a poly-G motif, wherein the poly-G motif optionally contains at least four Gs in a row or two G trimers, wherein the **CpG**-S motifs comprise motifs having the formula 5'X_{1CGX23}' wherein C is unmethylated, at least one nucleotide separates consecutive CG dinucleotides in. . . of adenine, guanine, and thymine and X_2 is selected from the group

consisting of cytosine, thymine, and adenine, wherein the **CpG**-N motifs are removed from non-essential regions of the nucleic acid construct and the **CpG**-S motifs are inserted into non-essential regions of the nucleic acid construct.

96. The method of claim 85, wherein the **CpG**-N motifs are selected from the group consisting of clusters of direct repeats of **CpG** dinucieotides, CCG trinucleotides; CGG trinucleotides, CCGG tetranuclootides, CGCG tetranucleotides and a combination thereof.

99. The method of claim 85, wherein the **CpG**-S motifs in the itmmunostimulatory nucleic acid construct comprise a **CpG** motif having the formula: 5'X_{1CGX23}' wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and.

. .

100. The method of claim 99, wherein the **CpG**-S motif is selected from the group consisting of GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT.

101. The method of claim 99, wherein the **CpG**-S motif comprises GTCGYT or TGACGTT.

102. The method of claim 99, wherein the **CpG**-S motif comprises TGTCGYT.

103. The method of claim 99, wherein the **CpG**-S motif comprises TCCATGTCGTTCCTGTCGTT (SEQ ID NO:1).

104. The method of claim 99, wherein the **CpG**-S motif comprises TCCTGACGTTCCTGACGTT (SEQ ID NO:2).

105. The method of claim 99, wherein the **CpG**-S motif comprises TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:3).

106. The method of claim 99, wherein the **CpG**-S motif comprises TCAACGTT.

L15 ANSWER 7 OF 12 USPATFULL on STN

2001:79141 **Immunostimulatory** nucleic acid molecules.

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US 6239116 B1 20010529

APPLICATION: US 1997-960774 19971030 (8)

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed:

1. A method for inducing Il-6 in a subject comprising administering to the subject an effective amount to induce Il-6 in the subject of an **immunostimulatory** nucleic acid, having a sequence comprising: 5'X_1 X_2 CGX_3 X3' wherein C is unmethylated, wherein X_1, X_2 and X_3, X_4 are nucleotides, and wherein the 5' X_1 X_2 CGX_3 X_4 3' sequence is a non-palindromic sequence.

2. The method of claim 1, wherein the subject is human.

3. The method of claim 1, wherein the nucleic acid has 8 to 100 nucleotides.

4. The method of claim 1, wherein the nucleic acid backbone includes a phosphate backbone modification on the 5' inter-nucleotide linkages.

5. The method of claim 1, wherein the nucleic acid backbone includes a phosphate backbone modification on the 3' inter-nucleotide linkages.

6. The method of claim 1, wherein the nucleic acid includes a phosphate backbone modification.

7. The method of claim 1, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, GpT, GpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

8. The method of claim 1, wherein X_1 X_2 are GpA and X_3

X_4 are TpT.

9. The method of claim 1, wherein X_1 and X_2 are purines and X_3 and X_4 are pyrimidines.

10. The method of claim 1, wherein X_1 X_2 are GpA and X_3 and X_4 are pyrimidines.

11. The method of claim 1, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

12. The method of claim 1, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and N is a nucleic acid sequence composed of from about 2-25 nucleotides.

13. The method of claim 1, wherein the **immunostimulatory** nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCCATGTCGCTCCTGATGCT (SEQ ID NO:37); TCCATAACGTTCCTGATGCT (SEQ ID NO:2); TCCATGACGATCCTGATGCT (SEQ ID NO:87); TCCATGGCGGTCCTGATGCT (SEQ ID NO:34); TCCATGTCGGTCCTGATGCT (SEQ ID NO:28); TCCATAACGTCCCTGATGCT (SEQ ID NO:88); TCCATGTCGTTCCTGATGCT (SEQ ID NO:38); and TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:46).

14. A method of stimulating natural killer cell lytic activity comprising exposing a natural killer cell to an **immunostimulatory** nucleic acid to stimulate natural killer cell lytic activity, the **immunostimulatory** nucleic acid having a sequence comprising: 5'X_1 X_2 CGX_3 X3' wherein C is unmethylated, wherein X_1 X_2 and X_3 X_4 are nucleotides, and wherein the 5'X_1 X_2 CGX_3 X_4 3' sequence is a non-palindromic sequence.

15. The method of claim 14, wherein the nucleic acid has 8 to 100 nucleotides.

16. The method of claim 14, wherein the nucleic acid backbone includes a phosphate backbone modification on the 5' inter-nucleotide linkages.

17. The method of claim 14, wherein the nucleic acid backbone includes a phosphate backbone modification on the 3' inter-nucleotide linkages.

18. The method of claim 14, wherein the nucleic acid includes a phosphate backbone modification.

19. The method of claim 14, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

20. The method of claim 14, wherein X_1 X_2 are GpA and X_3 X_4 are TpT.

21. The method of claim 14, wherein X_1 and X_2 are purines and X_3 and X_4 are pyrimidines.

22. The method of claim 14, wherein X_1 X_2 are GpA and X_3 and X_4 are pyrimidines.

23. The method of claim 14, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

24. The method of claim 15, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and N is a nucleic acid sequence composed of from about 2-25 nucleotides.

25. The method of claim 14, wherein the **immunostimulatory** nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCGTCGTTGTCGTTGTCGTT (SEQ ID NO:47); TCCATGACGGTCCTGATGCT (SEQ ID NO:35); TCCATGACGATCCTGATGCT (SEQ ID NO:87); TCCATGACGCTCCTGATGCT (SEQ ID NO:89); TCCATGACGTTCCTGATGCT (SEQ ID NO:7); TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:46); TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO:49); GCGTGCGTTGTCGTTGTCGTT (SEQ ID NO:56); TGTCGTTTGTCGTTTGTCGTT(SEQ ID NO:48); TGTCGTTGTCGTTGTCGTT (SEQ ID NO:50); and TCGTCGTCGTCGTT (SEQ ID NO:51).

26. A method for inducing interferon-gamma in a subject to treat an

immune system deficiency, comprising: administering to a subject having an immune system deficiency an effective amount to induce interferon-gamma production in the subject of an **immunostimulatory** nucleic acid, having a sequence comprising: 5'X_1 X_2 CGX_3 X_4 3' wherein C is unmethylated, wherein X_1 X_2 and X_3 X_4 are nucleotides, and wherein the sequence of the formula X_1 X_2 CGX_3 X_4 is not palindromic.

27. The method of claim 26, wherein the nucleic acid has 8 to 100 nucleotides.

28. The method of claim 26, wherein the nucleic acid backbone includes a phosphate backbone modification on the 5' inter-nucleotide linkages.

29. The method of claim 26, wherein the nucleic acid backbone includes a phosphate backbone modification on the 3' inter-nucleotide linkages.

30. The method of claim 26, wherein the nucleic acid includes a phosphates backbone modification.

31. The method of claim 26, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

32. The method of claim 26, wherein X_1 X_2 are GpA and X_3 X_4 are TpT.

33. The method of claim 26, wherein X_1 and X_2 are purines and X_3 and X_4 are pyrimidines.

34. The method of claim 26, wherein X_1 X_2 are GpA and X_3 and X_4 are pyrimidines.

35. The method of claim 26, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

36. The method of claim 26, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and N is a nucleic acid sequence composed of from about 2-25 nucleotides.

37. A method for inducing Il-12 in a subject comprising: administering to the subject an effective amount to induce Il-12 in the subject, of an **immunostimulatory** nucleic acid having a sequence comprising: 5'X_1 X_2 CGX_3 X3' wherein C is unmethylated, wherein X_1, X_2, X_3, and X_4 are nucleotides, and wherein the sequence of the formula X_1 X_2 CGX_3 X_4 is not palindromic.

38. The method of claim 37, wherein the subject is human.

39. The method of claim 37, wherein the **immunostimulatory** nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCCATGTCGCTCCTGATGCT (SEQ ID NO:37); TCCATGACGATCCTGATGCT (SEQ ID NO:87); TCCATGGCGGTCCTGATGCT (SEQ ID NO:34); TCCATGTCGGTCCTGATGCT (SEQ ID NO:28); TCCATAACGTCCCTGATGCT (SEQ ID NO:88); TCCATGTCGTTCCTGATGCT (SEQ ID NO:38); and TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:46).

40. The method of claim 37, wherein the nucleic acid has 8 to 100 nucleotides.

41. The method of claim 37, wherein the nucleic acid backbone includes a phosphate backbone modification on the 5' inter-nucleotide linkages.

42. The method of claim 37, wherein the nucleic acid backbone includes a phosphate backbone modification on the 3' inter-nucleotide linkages.

43. The method of claim 37, wherein the nucleic acid includes a phosphate backbone modification.

44. The method of claim 37, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

45. The method of claim 37, wherein X_1 X_2 are GpA and X_3 X_4 are TpT.

46. The method of claim 37, wherein X_1 and X_2 are purines and X_3 and X_4 are pyrimidines.

47. The method of claim 37, wherein X_1 X_2 are GpA and X_3 and X_4 are pyrimidines.

48. The method of claim 37, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

49. The method of claim 37, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and N is a nucleic acid sequence composed of from about 2-25 nucleotides.

TI **Immunostimulatory** nucleic acid molecules

AI **US 1997-960774 19971030 (8)**

AB Nucleic acid sequences containing unmethylated **CpG** dinucleotides that modulate an immune response including stimulating a Th1 pattern of immune activation, cytokine production, NK lytic activity, and. . .

SUMM . . . present invention relates generally to oligonucleotides and more specifically to oligonucleotides which have a sequence including at least one unmethylated **CpG** dinucleotide which are **immunostimulatory**.

SUMM . . . cAMP response element, the CRE, the consensus form of which is the unmethylated sequence TGACGTC (binding is abolished if the **CpG** is methylated) (Iguchi-Ariga, S. M. M., and W. Schaffner: "**CpG** methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation". Genes & Develop. 3:612,. . .

SUMM The present invention is based on the finding that certain nucleic acids containing unmethylated cytosine-guanine (**CpG**) dinucleotides activate lymphocytes in a subject and redirect a subject's immune response from a Th2 to a Th1 (e.g. by. . . to produce Th1 cytokines, including IL-12, IFN-γ and GM-CSF). Based on this finding, the invention features, in one aspect, novel **immunostimulatory** nucleic acid compositions.

SUMM In one embodiment, the invention provides an isolated **immunostimulatory** nucleic acid sequence containing a **CpG** motif represented by the formula:

SUMM In another embodiment, the invention provides an isolated **immunostimulatory** nucleic acid sequence contains a **CpG** motif represented by the formula:

SUMM In another embodiment, autoimmune disorders are treated by inhibiting a subject's response to **CpG** mediated leukocyte activation. The invention provides administration of inhibitors of endosomal acidification such as bafilomycin a, chloroquine, and monensin to. . .

DRWD FIG. 1 B. Control phosphodiester oligodeoxynucleotide (ODN) 5' ATGGAAGGTCCAGTGTTCTC3' (SEQ ID No: 1) (.box-solid.) and two phosphodiester **CpG** ODN 5' ATCGACCTACGTGCGTTCTC3' (SEQ ID No: 2) (.diamond-solid.) and 5' TCCATAACGTTCCTGATGCT3' (SEQ ID No: 3) (.circle-solid.).

DRWD FIG. 1 C. Control phosphorothioate ODN 5' GCTAGATGTTAGCGT3' (SEQ ID No: 4) (.box-solid.) and two phosphorothioate **CpG** ODN 5' GAGAACGTCGACCTTCGAT3' (SEQ ID No: 5) (.diamond-solid.) and 5' GCATGACGTTGAGCT3' (SEQ ID No: 6) (.circle-solid.). Data present the mean±standard. . .

DRWD FIG. 2 is a graph plotting IL-6 production induced by **CpG** DNA in vivo as determined 1-8 hrs after injection. Data represent the mean from duplicate analyses of sera from two mice. BALB/c mice (two mice/group) were injected iv. with 100 µl of PBS (.quadrature.) or 200 µg of **CpG** phosphorothioate ODN 5' TCCATGACGTTCCTGATGCT3' (SEQ ID No: 7) (.box-solid.) or non-**CpG** phosphorothioate ODN 5' TCCATGAGCTTCCTGAGTCT3' (SEQ ID No: 8) (.diamond-solid.).

DRWD . . . periods after in vivo stimulation of BALB/c mice (two mice/group) injected iv with 100 µl of PBS, 200 µg of **CpG** phosphorothioate ODN 5' TCCATGACGTTCCTGATGCT3' (SEQ ID No: 7) or non-**CpG** phosphorothioate ODN 5' TCCATGAGCTTCCTGAGTCT3' (SEQ ID No: 8).

DRWD FIG. 4A is a graph plotting dose-dependent inhibition of **CpG**-induced IgM production by anti-IL-6. Splenic B-cells from DBA/2 mice were stimulated with **CpG** ODN 5' TCCAAGACGTTCCTGATGCT3' (SEQ ID No: 9) in the presence of the indicated concentrations of neutralizing anti-IL-6 (.diamond-solid.) or isotype control Ab (.circle-solid.) and IgM levels in culture supernatants determined by ELISA. In the absence of **CpG** ODN, the anti-IL-6 Ab had no effect on IgM secretion (.box-solid.).

DRWD FIG. 4B is a graph plotting the stimulation index of **CpG**-induced

splenic B cells cultured with anti-IL-6 and **CpG** S-ODN 5' TCCATGACGTTCCTGATGCT3' (SEQ ID No: 7) (.diamond-solid.) or anti-IL-6 antibody only (.box-solid.). Data present the meanstandard deviation of triplicates.

DRWD . . . cells transfected with a promoter-less CAT construct (pCAT), positive control plasmid (RSV), or IL-6 promoter-CAT construct alone or cultured with **CpG** 5' CCATGACGTTCCTGATGCT3' (SEQ ID No: 7) or non-**CpG** 5' TCCATGAGCTTCCTGAGTCT3' (SEQ ID No: 8) phosphorothioate ODN at the indicated concentrations. Data present the mean of triplicates.

DRWD FIG. 6 is a schematic overview of the immune effects of the **immunostimulatory** unmethylated **CpG** containing nucleic acids, which can directly activate both B cells and monocytic cells (including macrophages and dendritic cells) as shown. The **immunostimulatory** oligonucleotides do not directly activate purified NK cells, but render them competent to respond to IL-12 with a marked increase in their IFN-γ production. By inducing IL-12 production and the subsequent increased IFN-γ secretion by NK cells, the **immunostimulatory** nucleic acids promote a Th1 type immune response. No direct activation of proliferation of cytokine secretion by highly purified T cells has been found. However, the induction of Th1 cytokine secretion by the **immunostimulatory** oligonucleotides promotes the development of a cytotoxic lymphocyte response.

DRWD FIG. 7 is an autoradiograph showing NFkB mRNA induction in monocytes treated with E. coli (EC) DNA (containing unmethylated **CpG** motifs), control (CT) DNA (containing no unmethylated **CpG** motifs) and lipopolysaccharide (LPS) at various measured times, 15 and 30 minutes after contact.

DRWD . . . the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the **CpG** oligo (**TCCATGACGTTCCTGACGTT** SEQ ID No. 10) also showed an increase in the level of reactive oxygen species such that more than 50%. . .

DRWD . . . have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with **CpG** DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and. . .

DRWD . . . egg antigen "SEA" (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given **CpG** oligo (SEQ ID NO. 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation. . .

DRWD . . . and subsequently inhale SEA (open circle), many eosinophils are present in the lungs. However, when the mice are initially given **CpG** oligo (SEQ ID NO. 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation. . .

DRWD . . . inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a **CpG** oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes. . .

DRWD . . . or SEQ ID No. 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can actually redirect the cytokine response of the lung to production of IL-12, indicating a Th1 type of immune. . .

DRWD . . . or SEQ ID No. 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can also redirect the cytokine response of the lung to production of IFN-g, indicating a Th1 type of immune. . .

DETD An "**immunostimulatory** nucleic acid molecule" refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (i.e. "**CpG** DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates (e.g. has a mitogenic effect on, or induces or increases cytokine expression by) a vertebrate lymphocyte. An **immunostimulatory** nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have. . .

DETD In one preferred embodiment the invention provides an isolated immunostimulalory nucleic acid sequence containing a **CpG** motif represented by the formula:

DETD In another embodiment the invention provides an isolated **immunostimulatory** nucleic acid sequence contains a **CpG** motif represented by the formula:

DETD Preferably the **immunostimulatory** nucleic acid sequences of the invnetion include X_1 X_2 selected from the group consisting of GpT, GpG, GpA and ApA. . . selected from the group consisting of TpT, CpT and GpT (see for example, Table 5). For facilitating uptake into cells, **CpG** containing **immunostimulatory** nucleic acid molecules are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size (even many kb long) are **immunostimulatory** if sufficient **immunostimulatory** motifs are present, since such larger

nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not. . . or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus mitogenic **CpG** motif is not a palindrome. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, where the oligonucleotide incorporates a phosphate backbone. . .

DETD Preferably the **immunostimulatory CpG** DNA is in the range of between 8 to 30 bases in size when it is an oligonucleotide. Alternatively, **CpG** dinucleotides can be produced on a large scale in plasmids, which after being administered to a subject are degraded into oligonucleotides. Preferred **immunostimulatory** nucleic acid molecules (e.g. for use in increasing the effectiveness of a vaccine or to treat an immune system deficiency. . .

DETD . . . useful as an adjuvant for use during antibody production in a mammal. Specific, but non-limiting examples of such sequences include: **TCCATGACGTTCCTGACGTT** (SEQ ID NO.10), GTCG(T/C)T and TGTCG(T/C)T. Furthermore, the claimed nucleic acid sequences can be administered to treat or prevent the symptoms of an asthmatic disorder by redirecting a subject's immune response from Th2 to Th1. An exemplary sequence includes **TCCATGACGTTCCTGACGTT** (SEQ ID NO.10).

DETD The stimulation index of a particular **immunostimulatory CpG** DNA can be tested in various immune cell assays. Preferably, the stimulation index of the **immunostimulatory CpG** DNA with regard to B-cell proliferation is at least about 5, preferably at least about 10, more preferably at least. . . treat an immune system deficiency by stimulating a cell-mediated (local) immune response in a subject, it is important that the **immunostimulatory CpG** DNA be capable of effectively inducing cytokine secretion by monocytic cells and/or Natural Killer (NK) cell lytic activity.

DETD Preferred **immunostimulatory CpG** nucleic acids should effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml. . . IL-6, 200 pg/ml IL-12, depending on the therapeutic indication, as determined by the assays described in Example 12. Other preferred **immunostimulatory CpG** DNAs should effect at least about 10%, more preferably at least about 15% and most preferably at least about 20%. . .

DETD . . . in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated **CpG** containing nucleic acid molecules that are tens to hundreds of kbs long are relatively resistant to in vivo degradation. For shorter **immunostimulatory** nucleic acid molecules, secondary structure can stabilize and increase their effect. For example, if the 3' end of a nucleic. . .

DETD . . . acid molecules (including phosphorodithioate-modified) can increase the extent of immune stimulation of the nucleic acid molecule, which contains an unmethylated **CpG** dinucleotide as shown herein. International Patent Application Publication Number: WO 95/26204 entitled "Immune Stimulation By Phosphorothioate Oligonucleotide Analogs" also reports on the non-sequence specific **immunostimulatory** effect of phosphorothioate modified oligonucleotides. As reported herein, unmethylated **CpG** containing nucleic acid molecules having a phosphorothioate backbone have been found to preferentially activate B-cell activity, while unmethylated **CpG** containing nucleic acid molecules having a phosphodiester backbone have been found to preferentially activate monocytic (macrophages, dendritic cells and monocytes) and NK cells. Phosphorothioate **CpG** oligonucleotides with preferred human motifs, are also strong activators of monocytic and NK cells.

DETD Certain Unmethylated **CpG** Containing Nucleic Acids Have B Cell Stimulatory Activity As Shown in vitro and in vivo

DETD . . . the other nonstimulatory control ODN. In comparing these sequences, it was discovered that all of the four stimulatory ODN contained **CpG** dinucleotides that were in a different sequence context from the nonstimulatory control.

DETD To determine whether the **CpG** motif present in the stimulatory ODN was responsible for the observed stimulation, over 300 ODN ranging in length from 5 to 42 bases that contained methylated, unmethylated, or no **CpG** dinucleotides in various sequence contexts were synthesized. These ODNs, including the two original "controls" (ODN 1 and 2) and two. . . then examined for in vitro effects on spleen cells (representative sequences are listed in Table 1). Several ODN that contained **CpG** dinucleotides induced B cell activation and IgM secretion; the magnitude of this stimulation typically could be increased by adding more **CpG** dinucleotides (Table 1; compare ODN 2 to 2a or 3D to 3Da and 3Db). Stimulation did not appear to result. . .

DETD Mitogenic ODN sequences uniformly became nonstimulatory if the **CpG** dinucleotide was mutated (Table 1; compare ODN 1 to 1a; 3D to 3Dc; 3M to 3Ma; and 4 to 4a) or if the cytosine of the **CpG** dinucleotide was replaced by 5-methylcytosine (Table 1; ODN 1b,2b,3Dd, and 3Mb). Partial

methylation of **CpG** motifs caused a partial loss of stimulatory effect (compare 2a to 2c, Table 1). In contrast, methylation of other cytosines did not reduce ODN activity (ODN 1c, 2d, 3De and 3Mc). These data confirmed that a **CpG** motif is the essential element present in ODN that activate B cells.

DETD In the course of these studies, it became clear that the bases flanking the **CpG** dinucleotide played an important role in determining the murine B cell activation induced by an ODN. The optimal stimulatory motif was determined to consist of a **CpG** flanked by two 5' purines (preferably a GpA dinucleotide) and two 3' pyrimidines (preferably a TpT or TpC dinucleotide). Mutations of ODN to bring the **CpG** motif closer to this ideal improved stimulation (e.g. Table 1, compare ODN 2 to 2e; 3M to 3Md) while mutations. . . Table 1, compare ODN 3D to 3Df; 4 to 4b, 4c and 4d). On the other hand, mutations outside the **CpG** motif did not reduce stimulation (e.g. Table 1, compare ODN 1 to id; 3D to 3Dg; 3M to 3Me). For. . .

DETD . . . 10 As. The effect of the G-rich ends is cis; addition of an ODN with poly G ends, but no **CpG** motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated **CpG** were found to be more **immunostimulatory**.

DETD . . . dev. derived from at least 3 separate experiments, and are compared to wells cultured with no added ODN.

ND = not done.

CpG dinucleotides are underlined.

Dots indicate identity; dashes indicate deletions.

Z indicates 5 methyl cytosine.

DETD TABLE 2

Identification of the optimal **CpG** motif for Murine IL-6 production and B cell activation

ODN	SEQUENCE (5'-3')	IL-6 (pg/ml)[a] CH12.LX	SPLENIC B CELL	SI[b]	IgM (ng/ml)[c]
512. . .	0.2	3534 ± 217			
1708	(SEQ ID No:106) .....CA..TG.....		ND	59 ± 3	1.5 ± 0.1
		466 ± 109			

Dots indicate identity; **CpG** dinucleotides are underlined; ND = not done

[a] The experiment was done at least three times with similar results. The level. . . CH12.LX and splenic B cells was ≤ 10 pg/ml. The IgM level of unstimulated culture was 547 ± 82 ng/ml. **CpG** dinucleotides are underlined and dots indicate identity.

[b] [3 H] Uridine uptake was indicated as a fold increase (SI: stimulation index) from unstimulated control (2322.67 ± 213.68 cpm). Cells were stimulated with 20 μM of various **CpG** O-ODN. Data present the mean ± SD of triplicates

[c] Measured by ELISA.

DETD . . . the subsequent fall in stimulation when purified B cells with or without anti-IgM (at a submitogenic dose) were cultured with **CpG** ODN, proliferation was found to synergistically increase about 10-fold by the two mitogens in combination after 48 hours. The magnitude. . .

DETD Cell cycle analysis was used to determine the proportion of B cells activated by **CpG**-ODN. **CpG**-ODN induced cycling in more than 95% of B cells. Splenic B lymphocytes sorted by flow cytometry into CD23- (marginal zone). . . as were both resting and activated populations of B cells isolated by fractionation over Percoll gradients. These studies demonstrated that **CpG**-ODN induce essentially all B cells to enter the cell cycle.

DETD **Immunostimulatory** Nucleic Acid Molecules Block Murine B Cell Apoptosis

DETD . . . are rescued from this growth arrest by certain stimuli such as LPS and by the CD40 ligand. ODN containing the **CpG** motif were also found to protect WEHI-231 from anti-IgM induced growth arrest, indicating that accessory cell populations are not required for the effect. Subsequent work indicates that **CpG** ODN induce Bcl-x and myc expression, which may account for the protection from apoptosis. Also, **CpG** nucleic acids have been found to block apoptosis in human cells. This inhibition of apoptosis is important, since it should enhance and prolong immune activation by **CpG** DNA.

DETD Identification of the optimal **CpG** motif for induction of Murine IL-6 and IgM secretion and B cell proliferation

DETD To evaluate whether the optimal B cell stimulatory **CpG** motif was identical with the optimal **CpG** motif for IL-6 secretion, a panel of ODN in which the bases flanking the **CpG** dinucleotide were progressively substituted was studied. This ODN panel was analyzed for effects on B cell proliferation, Ig production, and. . . using both splenic B cells and CH12.LX cells. As shown in Table 2, the optimal stimulatory motif contains an unmethylated **CpG** flanked by two 5' purines and two 3' pyrimidines. Generally a mutation of either 5' purine to pyrimidine or 3'. . . had less marked effects. Based on analyses of these and scores of other ODN, it was determined that the optimal

CpG motif for induction of IL-6 secretion is TGACGTT, which is identical with the optimal mitogenic and IgM-inducing **CpG** motif (Table 2). This motif was more stimulatory than any of the palindrome containing sequences studied (1639, 1707 and 1708).

DETD Induction of Murine Cytokine Secretion by **CpG** motifs in Bacterial DNA or Oligonucleotides

DETD As described in Example 9, the amount of IL-6 secreted by spleen cells after **CpG** DNA stimulation was measured by ELISA. T cell depleted spleen cell cultures rather than whole spleen cells were used for in vitro studies following preliminary studies showing, that T cells contribute little or nothing to the IL-6 produced by **CpG** DNA-stimulated spleen cells. As shown in Table 3, IL-6 production was markedly increased in cells cultured with E. coli DNA. . . response to bacterial DNA. To analyze whether the IL-6 secretion induced by E. coli DNA was mediated by the unmethylated **CpG** dinucleotides in bacterial DNA, methylated E. coli DNA and a panel of synthetic ODN were examined. As shown in Table 3, **CpG** ODN significantly induced IL-6 secretion (ODN 5a, 5b, 5c) while **CpG** methylated E. coli DNA, or ODN containing methylated **CpG** (ODN 5f) or no **CpG** (ODN 5d) did not. Changes at sites other than **CpG** dinucleotides (ODN 5b) or methylation of other cytosines (ODN 5g) did not reduce the effect of **CpG** ODN. Methylation of a single **CpG** in an ODN with three CpGs resulted in a partial reduction in the stimulation (compare ODN 5c to 5e; Table. . .

DETD TABLE 3

Induction of Murine IL-6 secretion by **CpG** motifs in bacterial DNA or oligonucleotides.

Treatment	IL-6 (pg/ml)
calf thymus DNA	≤10
calf thymus DNA + DNase	≤10
E. coli DNA	1169.5 ± 94.1
E. coli DNA + DNase	≤10
CpG methylated E. coli DNA	≤10
LPS	280.1 ± 17.1
Media (no DNA)	≤10

ODN			
5a	SEQ. ID. No:1	ATGGACTCTCCAGCGTTCTC	1096.4 ± 372.0

5b. . . or without enzyme treatment, or LPS (10 μg/ml) for 24 hr. Data represent the mean (pg/ml) ± SD of triplicates. **CpG** dinucleotides are underlined and dots indicate identity. Z indicates 5-methylcytosine.

DETD **CpG** motifs can be used as an artificial adjuvant

DETD . . . more acceptable side effects has led to the production of new synthetic adjuvants. The present invention provides the sequence 1826 **TCCATGACGTTCCTGACGTT** (SEQ ID NO: 10), which is an adjuvant including **CpG** containing nucleic acids. The sequence is a strong immune activating; sequence and is a superb adjuvant, with efficacy comparable or. . .

DETD Titration of induction of Murine IL-6 Secretion by **CpG** motifs

DETD Bacterial DNA and **CpG** ODN induced IL-6 production in T cell depleted murine spleen cells in a dose-dependent manner, but vertebrate DNA and non-**CpG** ODN did not (FIG. 1). IL-6 production plateaued at approximately 50 μg/ml of bacterial DNA or 40 μM of **CpG** O-ODN. The maximum levels of IL-6 induced by bacterial DNA and **CpG** ODN were 1-1.5 ng/ml and 2-4 ng/ml respectively. These levels were significantly greater than those seen after stimulation by LPS (0.35 ng/ml) (FIG. 1A). To evaluate whether **CpG** ODN with a nuclease-resistant DNA backbone would also induce IL-6 production, S-ODN were added to T cell depleted murine spleen cells. **CpG** S-ODN also induced IL-6 production in a dose-dependent manner to approximately the same level as **CpG** O-ODN while non-**CpG** S-ODN failed to induce IL-6 (FIG. 1C). **CpG** S-ODN at a concentration of 0.05 μM could induce maximal IL-6 production in these cells. This result indicated that the nuclease-resistant DNA backbone modification retains the sequence specific ability of **CpG** DNA to induce IL-6 secretion and that **CpG** S-ODN are more than 80-fold more potent than **CpG** O-ODN in this assay system.

DETD Induction of Murine IL-6 secretion by **CpG** DNA in vivo

DETD To evaluate the ability of bacterial DNA and **CpG** S-ODN to induce IL-6 secretion in vivo, BALB/c mice were injected iv. with 100 μg of E. coli DNA, calf thymus DNA, or **CpG** or non-stimulatory S-ODN and bled 2 hr after stimulation. The level of IL-6 in the sera from the E. coli. . . 13 ng/ml while IL-6 was not detected in the sera from calf thymus DNA or PBS injected groups (Table 4). **CpG** S-ODN also induced IL-6 secretion in vivo. The IL-6 level in the sera from **CpG** S-ODN injected groups was approximately 20 ng/ml. In contrast, IL-6 was not detected in the sera from non-stimulatory S-ODN stimulated. . .

DETD TABLE 4

Secretion of Murine IL-6 induced by **CpG** DNA stimulation in vivo.

Stimulant	IL-6 (pg/ml)
PBS	<50

E. coli DNA 13858 ± 3143
Calf Thymus DNA <50
CpG S-ODN 20715 ± 606
non-**CpG** S-ODN <50

Mice (2 mice/group) were i.v. injected with 100 µl of PBS, 200 µg of E. coli DNA or calf thymus DNA, or 500 µg of **CpG** S-ODN or non-**CpG** control S-ODN. Mice were bled 2 hr after injection and 1:10 dilution of each serum was analyzed by IL-6 ELISA. Sensitivity limit of IL-6 ELISA was 5 pg/ml. Sequences of the **CpG** S-ODN is 5'GCATGACGTTGAGCT3' (SEQ. ID. No: 6) and of the non-stimulatory S-ODN is 5'GCTAGATGTTAGCGT3' (SEQ. ID. No: 49). Note that although #there is a **CpG** in sequence 48, it is too close to the 3' end to effect stimulation, as explained herein. Data represent mean. . .

DETD Kinetics of Murine IL-6 secretion after stimulation by **CpG** motifs in vivo

DETD To evaluate the kinetics of induction of IL-6 secretion by **CpG** DNA in vivo, BALB/c mice were injected iv. with **CpG** or control non-**CpG** S-ODN. Serum IL-6 levels were significantly increased within 1 hr and peaked at 2 hr to a level of approximately 9 ng/ml in the **CpG** S-ODN injected group (FIG. 2). IL-6 protein in sera rapidly decreased after 4 hr and returned to basal level by 12 hr after stimulation. In contrast to **CpG** DNA stimulated groups, no significant increase of IL-6 was observed in the sera from the non-stimulatory S-ODN or PBS injected. . .

DETD Tissue distribution and kinetics of IL-6 mRNA expression induced by **CpG** moths in vivo

DETD As shown in FIG. 2, the level of serum IL-6 increased rapidly after **CpG** DNA stimulation. To investigate the possible tissue origin of this serum IL-6, and the kinetics of IL-6 gene expression in vivo after **CpG** DNA stimulation, BALB/c mice were injected iv with **CpG** or non-**CpG** S-ODN and RNA was extracted from liver, spleen, thymus, and bone marrow at various time points after stimulation. As shown. . . FIG. 3A, the level of IL-6 mRNA in liver, spleen, and thymus was increased within 30 min. after injection of **CpG** S-ODN. The liver IL-6 mRNA peaked at 2 hr post-injection and rapidly decreased and reached basal level 8 hr after. . . hr post-injection and then gradually decreased (FIG. 3A). IL-6 mRNA was significantly increased in bone marrow within 1 hr after **CpG** S-ODN injection but then returned to basal level. In response to **CpG** S-ODN, liver, spleen and thymus showed more substantial increases in IL-6 mRNA expression than the bone marrow.

DETD Patterns of Murine Cytokine Expression Induced by **CpG** DNA

DETD . . . within 30 minutes and the level of IL-6 increased strikingly within 2 hours in the serum of mice injected with **CpG** ODN. Increased expression of IL-12 and interferon gamma (IFN-γ) mRNA by spleen cells was also detected within the first two. . .

DETD TABLE 5

Induction of human PBMC cytokine secretion by **CpG** oligos

ODN	Sequence (5'-3')	IL-6[1]	TNF-α[1]	IFN-γ[1]	GM-CSF	IL-12
512	TCCATGTCGGTCCTGATGCT	500	140	15.6	70	250
SEQ ID NO:28						
1637	.....C.....	550	16	7.8.	. .	ID NO:3
1707	.....A..TC.....	300	70	17	0	70
SEQ ID NO:88						
1708	.....CA..TG.....	270	10	17	ND	10
SEQ ID NO:106						

dots indicate identity; **CpG** dinucleotides are underlined

[1] measured by ELISA using Quantikine kits from R&D Systems (pg/ml) Cells were cultured in 10% autologous serum. . .

DETD **CpG** DNA induces cytokine secretion by human PBMC, specifically monocytes

DETD . . . panels of ODN used for studying mouse cytokine expression were used to determine whether human cells also are induced by **CpG** motifs to express cytokine (or proliferate), and to identify the **CpG** motif(s) responsible. Oligonucleotide 1619 (GTCGTT; residues 6-11 of SEQ ID NO:105) was the best inducer of TNF-αand IFN-γ secretion, and. . . little induction of other cytokines. Thus, it appears that human lymphocytes, like murine lymphocytes, secrete cytokines differentially in response to **CpG** dinucleotides, depending on the surrounding bases. Moreover, the motifs that stimulate murine cells best differ from those that are most effective with human cells. Certain **CpG** oligodeoxynucleotides are poor at activating human cells (oligodeoxynucleotides 1707, 1708, which contain the palindrome forming sequences GACGTC; residues 6-11 of. . .

DETD . . . simply reflect a nonspecific death of all cell types Cytokine secretion in response to E. coli (EC) DNA requires unmethylated **CpG** motifs, since it is abolished by methylation of the EC DNA (next to the bottom row, Table 6). LPS contamination. . .

DETD TABLE 6

CpG DNA induces cytokine secretion by human PBMC

DNA	TNF-α(pg/ml)[1]	IL-6 (pg/ml)	IFN-γ (pg/ml)	RANTES (pg/ml)
EC DNA (50 µg/ml)	900	12,000.	.	.

cytokine production under these conditions was from monocytes (or other L-LME-sensitive cells).

[3] EC DNA was methylated using 2U/µg DNA of CpG methylase (New England Biolabs) according to the manufacturer's directions, and methylation confirmed by digestion with Hpa-II and Msp-I. As a. . .

DETD . . . cytokine production in the PBMC treated with L-LME suggested that monocytes may be responsible for cytokine production in response to CpG DNA. To test this hypothesis more directly, the effects of CpG DNA on highly purified human, monocytes and macrophages was tested. As hypothesized, CpG DNA directly activated production of the cytokines IL-6, GM-CSF, and TNF-α by human macrophages, whereas non-CpG DNA did not (Table 7).

DETD TABLE 7

CpG DNA induces cytokine expression in purified human macrophages

	IL-6 (pg/ml)	GM-CSF (pg/ml)	TNF-α(pg/ml)
Cells alone	0	0	0
CT DNA (50 µg/ml).	.	.	

DETD Biological Role of IL-6 in Inducing Murine IgM Production in Response to CpG Motifs

DETD The kinetic studies described above revealed that induction of IL-6 secretion, which occurs within 1 hr post CpG stimulation, precedes IgM secretion. Since the optimal CpG motif for ODN inducing secretion of IL-6 is the same as that for IgM (Table 2), whether the CpG motifs independently induce IgM and IL-6 production or whether the IgM production is dependent on prior IL-6 secretion was examined. The addition of neutralizing anti-IL-6 antibodies inhibited in vitro IgM production mediated by CpG ODN in a dose-dependent manner but a control antibody did not (FIG. 4A). In contrast, anti-IL-6 addition did not affect either the basal level or the CpG-induced B cell proliferation (FIG. 4B).

DETD Increased transcriptional activity of the IL-6promoter in response to CpG DNA

DETD The increased level of IL-6 mRNA and protein after CpG DNA stimulation could result from transcriptional or post-transcriptional regulation. To determine if the transcriptional activity of the IL-6 promoter was upregulated in B cells cultured with CpG ODN, a murine B cell line, WEHI-231, which produces IL-6 in response to CpG DNA, was transfected with an IL-6 promoter-CAT construct (pIL-6/CAT) (Pottratz, S. T. et al., 17B-estradiol) inhibits expression of human interleukin-6-promoter-reporter constructs by a receptor-dependent mechanism. J. Clin. Invest. 93:944). CAT assays were performed after stimulation with various concentrations of CpG or non-CpG ODN. As shown in FIG. 5, CpG ODN induced increased CAT activity in dose-dependent manner while non-CpG ODN failed to induce CAT activity. This confirms that CpG induces the transcriptional activity of the IL-6 promoter.

DETD Dependence of B cell activation by CpG ODN on the Number of 5' and 3' Phosphorothioate Internucleotide Linkages

DETD . . . DNA synthesis (by [3] H thymidine incorporation) in treated spleen cell cultures (Example 10). O-ODN (0/0 phosphorothioate modifications) bearing a CpG motif caused no spleen cell stimulation unless added to the cultures at concentrations of at least 10 µM (Example 10).. . .

DETD Dependence of CpG-mediated lymphocyte activation on the type of backbone modification

DETD . . . result from the nuclease resistance of the former. To determine the role of ODN nuclease resistance in immune stimulation by CpG ODN, the stimulatory effects of chimeric ODN in which the 5' and 3' ends were rendered nuclease resistant with either. . .

DETD . . . while the S-ODN with the 3D sequence was less potent than the corresponding S-O-ODN (Example 10). In comparing the stimulatory CpG motifs of these two sequences, it was noted that the 3D sequence is a perfect match for the stimulatory motif in that the CpG is flanked by two 5' purines and two 3' pyrimidines. However, the bases immediately flanking the CpG in ODN 3D are not optimal; it has a 5' pyrimidine and a 3' purine. Based on further testing, it. . . for immune stimulation is more stringent for S-ODN than for S-O- or O-ODN. S-ODN with poor matches to the optimal CpG motif cause little or no lymphocyte activation (e.g. Sequence 3D). However, S-ODN with good matches to the motif, most critically at the positions immediately flanking the CpG, are more potent than the corresponding S-O-ODN (e.g. Sequence 3M, Sequences 4 and 6), even though at higher concentrations (greater. . .

DETD The increased B cell stimulation seen with CpG ODN bearing S or S_2 substitutions could result from any or All of the following effects: nuclease resistance, increased cellular. . . However, nuclease resistance can not be the only explanation, since the MP-O-ODN were actually less stimulatory than the O-ODN with CpG motifs. Prior

studies have shown that ODN uptake by lymphocytes is markedly affected by the backbone chemistry (Zhao et al.,. . .

DETD Unmethylated **CpG** Containing Oligos Have NK Cell Stimulatory Activity

DETD Experiments were conducted to determine whether **CpG** containing oligonucleotides stimulated the activity of natural killer (NK) cells in addition to B cells. As shown in Table 8, a marked induction of NK activity among spleen cells cultured with **CpG** ODN 1 and 3Dd was observed. In contrast, there was relatively no induction in effectors that had been treated with non-**CpG** control ODN.

DETD TABLE 8

Induction Of NK Activity By **CpG** Oligodeoxynucleotides (ODN)

	% YAC-1 Specific Lysis* Effector: Target		% 2C11 Specific Lysis Effector: Target	
ODN	50:1	100:1	50:1	100:1
None	-1.1 -1.4	15.3	16.6	
1	16.1	24.5	38.7	47.2
3Dd	17.1	27.0	37.0	40.0
non-**CpG** ODN	-1.6 -1.7	14.8	15.4	

DETD Induction of NK activity by DNA containing **CpG** motifs, but not by non-**CpG** DNA

DETD . . . 9). To determine whether the stimulatory activity of bacterial DNA may be a consequence of its increased level of unmethylated **CpG** dinucleotides, the activating properties of more than 50 synthetic ODN containing unmethylated, methylated, or no **CpG** dinucleotides was tested. The results, summarized in Table 9, demonstrate that synthetic ODN can stimulate significant NK activity, as long as they contain at least one unmethylated **CpG** dinucleotide. No difference was observed in the stimulatory effects of ODN in which the **CpG** was within a palindrome (such as ODN 1585, which contains the palindrome AACGTT) from those ODN without palindromes (such as 1613 or 1619), with the caveat that optimal stimulation was generally seen with ODN in which the **CpG** was flanked by two 5' purines or a 5' GpT dinucleotide and two 3' pyrimidines. Kinetic experiments demonstrated that NK. . . of the ODN. The data indicates that the murine NK; response is dependent on the prior activation of monocytes by **CpG** DNA, leading to the production of IL-12, TNF-α, and IFN-α/b (Example 11).

DETD TABLE 9

Induction of NK Activity by DNA Containing **CpG** Motifs but not by Non-**CpG** DNA

DNA or Cytokine Added		LU/10⁶ Mouse Cells	Human Cells
Expt. 1 None		0.00	0.00
IL-2		16.68	15.82
E.Coli. DNA		7.23	5.05
. . . No.42) 5.22			
1759 -------Z--*--	(SEQ ID No.52)	0.02	ND
1613 TCCATGTCGTTCCTGATGCT	(SEQ ID No:38)	3.35	
1765 -------Z---------	(SEQ ID No.53)	0.11	

CpG dinucleotides in ODN sequences are indicated by underlying; Z indicates methylcytosine. Lower case letters indicate nuclease resistant phosphorothioate modified internucleotide. . .

DETD From all of these studies, a more complete understanding of the immune, effects of **CpG** DNA has been developed, which is summarized in FIG. 6.

DETD Immune activation by **CpG** motifs may depend on bases flanking the **CpG**, and the number and spacing of the CpGs present within an ODN. Although a single **CpG** in an ideal base context can be a very strong and useful immune activator, superior effects can be seen with ODN containing several CpGs with the appropriate spacing and flanking bases. For activation of murine B cells, the optimal **CpG** motif is TGACGTT; residues 10-17 of SEQ ID NO:70.

DETD . . . ODN sequences for stimulation of human cells by examining the effects of changing the number, spacing, and flanking bases of **CpG** dinucleotides.

DETD Identification of phosphorothioate ODN with optimal **CpG** motifs for activation of human NK cells

DETD . . . cellular uptake (Krieg et al., Antisense Res. Dev. 6:133, 1996.) and improved B cell stimulation if they also have a **CpG** motif. Since NK activation correlates strongly with in vivo adjuvant effects, the identification of phosphorothioate ODN that will activate human. .

DETD The effects of different phosphorothioate ODNs--containing **CpG** dinucleotides in various base contexts--on human NK activation (Table 10) were examined. ODN 1840, which contained 2 copies of the. . . 10). To further identify additional ODNs optimal for NK activation, approximately one hundred ODN containing different numbers and spacing of **CpG** motifs, were tested with ODN 1982 serving as a control. The results are shown in Table 11.

DETD . . . ODNs began with a TC or TG at the 5' end, however, this

requirement was not mandatory. ODNs with internal **CpG** motifs (e.g. ODN 1840) are generally less potent stimulators than those in which a GTCGCT motif (residues 3-8 of SEQ. . . in which only one of the motifs had the addition of the spacing two Ts. The minimal acceptable spacing between **CpG** motifs is one nucleotide as long as the ODN has two pyrimidines (preferably T) at the 3' end (e.g., ODN. . . T also created a reasonably strong inducer of NK activity (e.g., ODN 2016). The choice of thymine (T) separating consecutive **CpG** dinucleotides is not absolute, since ODN 2002 induced appreciable NK activation despite the fact that adenine (A) separated its CpGs (i.e., CGACGTT; residues 14-20 of SEQ ID NO:82). It should also be noted that ODNs containing no **CpG** (e.g., ODN 1982), runs of CpGs, or CpGs in bad sequence contexts (e.g., ODN 2010) had no stimulatory effect on. . .

DETD TABLE 11

Induction of NK LU by Phoshorothioate **CpG** ODN with Good Motifs

ODN[1]				
cells		expt. 1	expt. 2	expt. 3
alone	sequence (5'-3') SEQ ID NO:	0.00	1.26	0.46
1840	TCCATGTCGTTCCTGTCGTT 73. . .			

(SEQ ID NO:83); Z = 5-methyl cytosine at residues 8 and 17; LU is lytic units; ND = not done; **CpG** dinucleotides are underlined for clarity

DETD Identification of phosphorothioate ODN with optimal **CpG** motifs for activation of human B cell proliferation

DETD The ability of a **CpG** ODN to induce B cell proliferation is a good measure of its adjuvant potential. Indeed, ODN with strong adjuvant effects generally also induce B cell proliferation. To determine whether the optimal **CpG** ODN for inducing B cell proliferation are the same as those for inducing NK cell activity, similar panels of ODN. . .

DETD TABLE 12

Induction of human B cell proliferation by Phosphorothioate **CpG** ODN

		Stimulation Index[1L]			
ODN	sequence (5' 3') SEQ ID NO:	expt. 1	expt. 2	expt. 3	expt. 4

expt. 5 expt.. . .

DETD The ability of a **CpG** ODN to induce IL-12 secretion is a good measure of its adjuvant potential, especially in terms of its ability to. . . IL-12 secretion from human PBMC in vitro (Table 13) was examined. These experiments showed that in some human PBMC, most **CpG** ODN could induce IL-12 secretion (e.g., expt. 1). However, other donors responded to just a few **CpG** ODN (e.g., expt. 2). ODN 2006 was a consistent inducer of IL2 secretion from most subjects (Table 13).

DETD TABLE 13

Induction of human IL-12 secretion by Phosphorothioate **CpG** ODN

		SEQ	IL-12 (pg/ml)	
		ID	expt.	expt.
ODN[1]	sequence (5'-3')	NO	2	
cells		0	0	
alone				
1962	TCCTGTCGTTCCTTGTCGTT	52	19	0
1965	TCCTGTCGTTTTTTGTCGTT	53. . .		

DETD As shown in FIG. 6, **CpG** DNA can directly activate highly purified B cells and monocytic cells. There are many similarities in the mechanism through which **CpG** DNA activates these cell types. For example, both require NFkB activation as explained further below.

DETD In further studies of different immune effects of **CpG** DNA, it was found that there is more than one type of **CpG** motif. Specifically, oligo 1668, with the best mouse B cell motif, is a strong inducer of both B cell and. . .

DETD TABLE 14

Different **CpG** motifs stimulate optimal murine B cell and NK activation

ODN	Sequence		B cell activation[1]	NK activation[2]
1668	TCCATGACGTTCCTGATGCT	(SEQ.ID.NO:7)	42,849	2.52
1758	TCTCCCAGCGTGCGCCAT	(SEQ.ID.NO.45)	1,747	6.66
NONE			367	0.00

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance. [1] Measured by [3] H. . .

DETD Teleological Basis of **Immunostimulatory**, Nucleic Acids

DETD Vertebrate DNA is highly methylated and **CpG** dinucleotides are underrepresented. However, the stimulatory **CpG** motif is common in microbial genomic DNA, but quite rare in vertebrate DNA. In addition, bacterial DNA has been reported. . . P. et al., J. Immunol. 147:1759 (1991)). Experiments further described in Example 3, in which methylation of bacterial DNA with **CpG** methylase was found to abolish mitogenicity, demonstrates that the difference in **CpG** status is the cause of B cell stimulation by bacterial DNA. This data supports the following conclusion: that unmethylated **CpG** dinucleotides present within bacterial DNA are responsible for the stimulatory effects of

bacterial DNA.

DETD Teleologically, it appears likely that lymphocyte activation by the **CpG** motif represents an immune defense mechanism that can thereby distinguish bacterial from host DNA. Host DNA, which would commonly be. . . regions and areas of inflammation due to apoptosis (cell death), would generally induce little or no lymphocyte activation due to **CpG** suppression and methylation. However, the presence of bacterial DNA containing unmethylated **CpG** motifs can cause lymphocyte activation precisely in infected anatomic regions, where it is beneficial. This novel activation pathway provides a rapid alternative to T cell dependent antigen specific B cell activation. Since the **CpG** pathway synergizes with B cell activation through the antigen receptor, B cells bearing antigen receptor specific for bacterial antigens would. . .

DETD . . . 35:647 (1992)), is likely triggered at least in part by activation of DNA-specific B cells through stimulatory signals provided by **CpG** motifs, as well as by binding of bacterial DNA to antigen receptors.

DETD . . . products released from dying bacteria that reach concentrations sufficient to directly activate many lymphocytes. Further evidence of the role of **CpG** DNA in the sepsis syndrome is described in Cowdery, J., et. al., (1996) The Journal of Immunology 156:4570-4575.

DETD Unlike antigens that trigger B cells through their surface Ig receptor, **CpG**-ODN did not induce any detectable Ca^{2+} flux, changes in protein tyrosine phosphorylation, or IP 3 generation. Flow cytometry with FITC-conjugated ODN with or without a **CpG** motif was performed sis described in Zhao, Q et al., (Antisense Research and Development 3:53-66 (1993)), and showed equivalent membrane binding, cellular uptake, efflux, and intracellular localization. This suggests that there may not be cell membrane proteins specific for **CpG** ODN. Rather than acting through the cell membrane, that data suggests that unmethylated **CpG** containing oligonucleotides require cell uptake for activity: ODN covalently linked to a solid Teflon support were nonstimulatory, as were biotinylated ODN immobilized on either avidin beads or avidin coated petri dishes. **CpG** ODN conjugated to either FITC or biotin retained full mitogenic properties, indicating no steric hindrance.

DETD Recent data indicate the involvement of the transcription factor NFkB as a direct or indirect mediator of the **CpG** effect. For example, within 15 minutes of treating B cells or monocytes with **CpG** DNA, the level of NFkB binding activity is increased (FIG. 7). However, it is not increased by DNA that does not contain **CpG** motifs. In addition, it was found that two different inhibitors of NFkB activation, PDTC and gliotoxin, completely block the lymphocyte stimulation by **CpG** DNA as measured by B cell proliferation or monocytic cell cytokine secretion, suggesting that NFkB activation is required for both. . .

DETD . . . various protein kinases, or through the generation of reactive oxygen species. No evidence for protein kinase activation induced immediately after **CpG** DNA treatment of B cells or monocytic cells have been found, and inhibitors of protein kinase A, protein kinase C, and protein tyrosine kinases had no effects on the **CpG** induced activation. However, **CpG** DNA causes a rapid induction of the production of reactive oxygen species in both B cells and monocytic cells, as. . . reactive oxygen species completely block the induction of NFkB and the later induction of cell proliferation and cytokine secretion by **CpG** DNA.

DETD Working backwards, the next question was how **CpG** DNA leads to the generation of reactive oxygen species so quickly. Previous studies by the inventors demonstrated that oligonucleotides and. . . rapidly become acidified inside the cell. To determine whether this acidification step may be important in the mechanism through which **CpG** DNA activates reactive oxygen species, the acidification step was blocked with specific inhibitors of endosome acidification including chloroquine, monensin, and. . . the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the **CpG** oligo also showed an increase in the level of reactive oxygen species such that more than 50% of the cells became positive (Panel D). However, cells treated with an oligonucleotide with the identical sequence except that the **CpG** was switched did not show this significant increase in the level of reactive oxygen species (Panel E).

DETD . . . have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with **CpG** DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and. . . This demonstrates that unlike the PMA plus ionomycin, the generation of reactive oxygen species following treatment of B cells with **CpG** DNA requires that the DNA undergo an acidification step in the endosomes. This is a completely novel mechanism of leukocyte activation. Chloroquine, monensin, and bafilomycin also appear to block the activation of NFkB by **CpG** DNA as well as the subsequent proliferation and induction of cytokine secretion.

DETD Chronic Immune Activation by **CpG** DNA and Autoimmune Disorders

DETD B cell activation by **CpG** DNA synergizes with signals through the B cell receptor. This raises the possibility that DNA-specific B cells may be activated by the concurrent binding of bacterial DNA to their antigen receptor, and by the co-stimulatory **CpG**-mediated signals. In addition, **CpG** DNA induces B cells to become resistant to apoptosis, a mechanism thought to be important for preventing immune responses to self antigens, such as DNA. Indeed, exposure to bDNA can trigger anti-DNA Ab production. Given this potential ability of **CpG** DNA to promote autoimmunity, it is therefore noteworthy that patients with the autoimmune disease systemic lupus erythematosus have persistently elevated. . . . circulating plasma DNA which is enriched in hypomethylated CpGs. These findings suggest a possible role for chronic immune activation by **CpG** DNA in lupus etiopathogenesis.

DETD . . . While the therapeutic mechanism of these drugs has been unclear, they are known to inhibit endosomal acidification. Leukocyte activation by **CpG** DNA is not mediated through binding to a cell surface receptor, but requires cell uptake, which occurs via adsorptive endocytosis into an acidified chloroquine-sensitive intracellular compartment. This suggested the hypothesis that leukocyte activation by **CpG** DNA may occur in association with acidified endosomes, and might even be pH dependent. To test this hypothesis specific inhibitors of DNA acidicification were applied to determine whether B cells or monocytes could respond to **CpG** DNA if endosomal acidification was prevented.

DETD The earliest leukocyte activation event that was detected in response to **CpG** DNA is the production of reactive oxygen species (ROS), which is induced within five minutes in primary spleen cells and. . . cell lines. Inhibitors of endosomal acidification including chloroquine, bafilomycin A, and monensin, which have different mechanisms of action, blocked the **CpG**-induced generation of ROS, but had no effect on ROS generation mediated by PMA, or ligation of CD40 or IgM. These. . . diverse pathways. This ROS generation is generally independent of endosomal acidification, which is required only for the ROS response to **CpG** DNA. ROS generation in response to **CpG** is not inhibited by the NFκB inhibitor gliotoxin, confirming that it is not secondary to NFκB activation.

DETD To determine whether endosomal acidification of **CpG** DNA was also required for its other immune stimulatory effects were performed. Both LPS and **CpG** DNA induce similar rapid NFκB activation, increases in proto-oncogene mRNA levels, and cytokine secretion. Activation of NFκB by DNA depended on **CpG** motifs since it was not induced by bDNA treated with **CpG** methylase, nor by ODN in which bases were switched to disrupt the CpGs. Supershift experiments using specific antibodies indicated that the activated NFκB complexes included the p50 and p65 components. Not unexpectedly, NFκB activation in LPS- or **CpG**-treated cells was accompanied by the degradation of IκBα and IκBβ. However, inhibitors of endosomal acidification selectively blocked all of the **CpG**-induced but none of the LPS-induced cellular activation events. The very low concentration of chloroquine (<10 μM) that has been determined to inhibit **CpG**-mediated leukocyte activation is noteworthy since it is well below that required for antimalarial activity and oiler reported immune effects (e.g., 100-1000 μM). These experiments support the role of a pH-dependent signaling mechanism in mediating the stimulatory effects of **CpG** DNA.

DETD TABLE 15

Specific blockade of **CpG**-induced TNF-α and IL-12 expression by inhibitors of endosomal acidification or NFκB activation

	Inhibitors:								NAC
	TPCK		Gliotoxin		Bisgliotoxin				(50
	Bafilomycin		Chloroquine		Monensin				. . .
	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	TNF-α	TNF-α	
Medium	37	147	46	102	27	20	22	73	10
	24	17	41						
CpG	455	17,114	71	116	28	6	49	777	54
	23	31	441						
ODN									
LPS	901	22,485	1370	4051	1025	12418	491	4796.	. .

were cultured with or without the indicated inhibitors at the concentrations shown for 2 hr and then stimulated with the **CpG** oligodeoxynucleotide (ODN) 1826 (**TCCATGACGTTCCTGACGTT** SEQ ID NO:10) at 2 μM or LPS (10 μg/ml) for 4 hr (TNF-α or 24 hr (IL-12) at which. . . Immunol., 157, 5394-5402 (1996); Krieg, A. M, J Lab. Clin. Med., 128, 128-133 (1996). Cells cultured with ODN that lacked **CpG** motifs did not induce cytokine secretion. Similar specific #inhibition of **CpG** responses was seen with IL-6 assays, and in experiments using primary spleen cells or the B cell lines CH12.LX and. . .

DETD Excessive immune activation by **CpG** motifs may contribute to the

pathogenesis of the autoimmune disease systemic lupus erythematosus, which is associated with elevated levels of circulating hypomethylated **CpG** DNA. Chloroquine and related antimalarial compounds are effective therapeutic agents for the treatment of systemic lupus erythematosus and some other. . . mechanism of action has been obscure. Our demonstration of the ability of extremely low concentrations of chloroquine to specifically inhibit **CpG**-mediated leukocyte activation suggests a possible new mechanism for its beneficial effect. It is noteworthy that lupus recurrences frequently are thought. . . bDNA present in infected tissues can be sufficient to induce a local inflammatory response. Together with the likely role of **CpG** DNA as a mediator of the sepsis syndrome and other diseases our studies suggest possible new therapeutic applications for antimalarial. . .

DETD **CpG**-induced ROS generation could be an incidental consequence of cell activation, or a signal that mediates this activation. The ROS scavenger N-acetyl-L-cysteine (NAC) blocks **CpG**-induced NFκB activation, cytokine production, and B cell proliferation, suggesting a causal role for ROS generation in these pathways. These data. . . gliotoxin (0.2 μg/ml). Cell aliquots were then cultured as above for 10 minutes in RPMI medium with or without a **CpG** ODN (1826) or non-**CpG** ODN (1911) at 1 μM or phorbol myristate acetate (PMA) plus ionomycin (iono). Cells were then stained with dihydrorhodamine-123 and. . . 5394-5402 (1996); Krieg, A. M, J. Lab. Clin. Med., 128, 128-133 (1996)). J774 cells, a monocytic line, showed similar pH-dependent **CpG** induced ROS responses. In contrast, **CpG** DNA did not induce the generation of extracellular ROS, nor any detectable neutrophil ROS. These concentrations of chloroquine (and those used with the other inhibitors of endosomal acidification) prevented acidification of the internalized **CpG** DNA using fluorescein conjugated ODN as described by Tonkinson, et al., (Nucl. Acids Res. 22, 4268 (1994); A. M. Krieg,. . .

DETD While NFκB is known to be an important regulator of gene expression, it's role in the transcriptional response to **CpG** DNA was uncertain. To determine whether this NFκB activation was required for the **CpG** mediated induction of gene expression cells were activated with **CpG** DNA in the presence or absence of pyrrolidine dithiocarbamate (PDTC), an inhibitor of IκB phosphorylation. These inhibitors of NFκB activation completely blocked the **CpG**-induced expression of protooncogene and cytokine mRNA and protein, demonstrating the essential role of NFκB as a mediator of these events.. . . was cultured in the presence of calf thymus (CT), E. coli (EC), or methylated E. coli (mEC) DNA (methylated with **CpG** methylase as described[4]) at 5 μg/ml or a **CpG** oligodeoxynucleotide (ODN 1826; Table 15) or a non-**CpG** ODN (ODN 1745; TCCATGAGCTTCCTGAGTCT; SEQ ID NO:8) at 0.75 μM for 1 hr, following which the cells were lysed and. . . was determined by supershifting with specific Ab to p65 and p50 (Santa Cruz Biotechnology, Santa Cruz, Calif.). Chloroquine inhibition of **CpG**-induced but not LPS-induced NFκB activation was established using J774 cells. The cells were precultured for 2 hr in the presence or absence of chloroquine (20 μg/ml) and then stimulated as above for 1 hr with either EC DNA, **CpG** ODN, non-**CpG** ODN or LPS (1 μg/ml). Similar chloroquine sensitive **CpG**-induced activation of NFκB was seen in a B cell line, WEHI-231 and primary spleen cells. These experiments were performed three. . .

DETD It was also established that **CpG**-stimulated mRNA expression requires endosomal acidification and NFκB activation in B cells and monocytes. J774 cells (2×10^6 cells/ml) were cultured for. . . stimulated with the addition of E. coli DNA (EC; 50 μg/ml), calf thymus DNA (CT; 50 μg/ml), LPS (10 μg/ml), **CpG** ODN (1826; 1 μM), or control non-**CpG** ODN (1911; 1 μM) for 3 hr. WEHI-231 B cells (5×10^5 cells/ml) were cultured in the presence or absence of gliotoxin (0.1 μg/ml) or bisgliotoxin (0.1 μg/ml) for 2 hrs and then stimulated with a **CpG** ODN (1826), or control non-**CpG** ODN (1911; TCCAGGACTTTCCTCAGGTT; SEQ ID NO:107) at 0.5 μM for 8 hr. In both cases, cells were harvested and RNA. . .

DETD The results indicate that leukocytes respond to **CpG** DNA through a novel pathway involving the pH-dependent generation of intracellular ROS. The pH dependent step may be the transport or processing of the **CpG** DNA, the ROS generation, or some other event. ROS are widely thought to be second messengers in signaling pathways in. . .

DETD Presumably, there is a protein in or near the endosomes that specifically recognizes DNA containing **CpG** motifs and leads to the generation of reactive oxygen species. To detect any protein in the cell cytoplasm that may specifically bind **CpG** DNA, electrophoretic mobility shift assays (EMSA) were used with 5' radioactively labeled oligonucleotides with or without **CpG** motifs. A band was found that appears to represent a protein binding specifically to single stranded oligonucleotides that have **CpG** motifs, but not to oligonucleotides that lack **CpG** motifs or to oligonucleotides in which the **CpG** motif has been methylated. This binding activity is blocked if excess of

oligonucleotides that contain the NFkB binding site was. . . suggests that an NFkB or related protein is a component of a protein or protein complex that binds the stimulatory **CpG** oligonucleotides.

DETD . . . points where NFkB was strongly activated. These data therefore do not provide proof that NFkB proteins actually bind to the **CpG** nucleic acids, but rather that the proteins are required in some way for the **CpG** activity. It is possible that a CREB/ATF or related protein may interact in some way with NFkB proteins or other proteins thus explaining the remarkable similarity in the binding motifs for CREB proteins and the optimal **CpG** motif. It remains possible that the oligos bind to a CREB/ATF or related protein, and that this leads to NFkB. . .

DETD Alternatively, it is very possible that the **CpG** nucleic acids may bind to one of the TRAF proteins that bind to the cytoplasmic region of CD40 and mediate. . .

DETD Method for Making **Immunostimulatory** Nucleic Acids

DETD . . . described (Uhlmann, E. and Peyman, A. (1990) Chem. Rev. 90:544; Goodchild, J. (1990) Bioconjugate Chem. 1:165). 2'-O-methyl nucleic acids with **CpG** motifs also cause immune activation, as do ethoxy-modified **CpG** nucleic acids. In fact, no backbone modifications have been found that completely abolish the **CpG** effect, although it is greatly reduced by replacing the C with a 5-methyl C.

DETD Therapeutic Uses of **Immunostimulatory** Nucleic Acid Molecules

DETD Based on their **immunostimulatory** properties, nucleic acid molecules containing at least one unmethylated **CpG** dinucleotide can be administered to a subject in vivo to treat an "immune system deficiency". Alternatively, nucleic acid molecules containing at least one unmethylated **CpG** dinucleotide can be contacted with lymphocytes (e.g. B cells, monocytic cells or NK cells) obtained from a subject having an. . .

DETD As reported herein, in response to unmethylated **CpG** containing nucleic acid molecules, an increased number of spleen cells secrete IL-6, IL-12, IFN-γ, IFN-α, IFN-β, IL-1, IL-3, IL-10, TNF-α,. . .

DETD **Immunostimulatory** nucleic acid molecules can also be administered to a subject in conjunction with a vaccine to boost a subject's immune system and thereby effect a better response from the vaccine. Preferably the **immunostimulatory** nucleic acid molecule is administered slightly before or at the same time as the vaccine. A conventional adjuvant may optionally. . .

DETD . . . antigen encoded by the vaccine determines the specificity of the immune response. Second, if the backbone of the plasmid contains **CpG** motifs, it functions as an adjuvant for the vaccine. Thus, **CpG** DNA acts as an effective "danger signal" and causes the immune system to respond vigorously to new antigens in the area. This mode of action presumably results primarily from the stimulatory local effects of **CpG** DNA on dendritic cells and other "professional" antigen presenting cells, as well as from the co-stimulatory effects on B cells.

DETD **Immunostimulatory** oligonucleotides and unmethylated **CpG** containing vaccines, which directly activate lymphocytes and co-stimulate an antigen-specific response, are fundamentally different from conventional adjuvants (e.g. aluminum precipitates),. . .

DETD In addition, an **immunostimulatory** oligonucleotide can be administered prior to, along with or after administration of a chemotherapy or immunotherapy to increase the responsiveness. . . chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF. **CpG** nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Induction of NK activity and. . .

DETD Another use of the described **immunostimulatory** nucleic acid molecules is in desensitization therapy for allergies, which are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated **CpG** nucleic acids, are predominantly of a class called "Th1" which is most marked by a cellular immune response and is. . . are mediated by Th2 type immune responses and autoimmune diseases by Th1 immune response. Based on the ability of the **immunostimulatory** nucleic acid molecules to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of an **immunostimulatory** nucleic acid (or a vector containing a nucleic acid) alone or in conjunction with an allergen can be administered to. . .

DETD Nucleic acids containing unmethylated **CpG** motifs may also have significant therapeutic utility in the treatment of asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated. . .

DETD As described in detail in the following Example 12, oligonucleotides containing an unethylated **CpG** motif (i.e., **TCCATGACGTTCCTGACGTT**; SEQ D NO. 10), but not a control oligonucleotide (TCCATGAGCTTCCTGAGTCT; SEQ ID NO 8) prevented the development of an inflammatory. . .

DETD For use in therapy, an effective amount of an appropriate **immunostimulatory** nucleic acid molecule alone or formulated as a delivery complex can be administered to a subject by any mode allowing. . .

DETD . . . to realize a desired biologic effect. For example, an effective amount of a nucleic acid containing at least one unmethylated **CpG** for treating an immune system deficiency could be that amount necessary to eliminate a tumor, cancer, or bacterial, viral or. . . such factors as the disease or condition being treated, the particular nucleic acid being administered (e.g. the number of unmethylated **CpG** motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or. . .

DETD . . . Table 1). In some experiments, culture supernatants were assayed for IgM by ELISA, and showed similar increases in response to **CpG**-ODN.

DETD . . . C57BL/6 spleen cells were cultured in two ml RPMI (supplemented as described for Example 1) with or without 40 μM **CpG** or non-**CpG** ODN for forty-eight hours. Cells were washed, and then used as effector cells in a short term ^{51} Cr release. . .

DETD In vivo Studies with **CpG** Phosphorothioate ODN

DETD B cells were cultured with phosphorothioate ODN with the sequence of control ODN 1a or the **CpG** ODN 1d and 3Db and then either pulsed after 20 hr with ^{3} H uridine or after 44 hr with. . .

DETD . . . for 1 hr. at 37 C. in the presence or absence of LPS or the control ODN 1a or the **CpG** ODN 1d and 3Db before addition of anti-IgM (1 μ/ml). Cells were cultured for a further 20 hr. before a. . .

DETD DBA/2 female mice (2 mos. old) were injected IP with 500 g **CpG** or control phosphorothioate ODN. At various time points after injection, the mice were bled. Two mice were studied for each. . .

DETD . . . (2U/μg of DNA) at 37° C. for 2 hr in 1×SSC with 5 mM MgCl2. To methylate the cytosine in **CpG** dinucleotides in E. coli DNA, E. coli DNA was treated with **CpG** methylase (M. SssI; 2 U/μg of DNA) in NEBuffer 2 supplemented with 160 μM S-adenosyl methionine and incubated overnight at. . .

DETD . . . humidified incubator maintained in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 1.5 mM L-glutamine, 50 μg/ml), **CpG** or non-**CpG** phosphodiester ODN (O-ODN) (20 μM), phosphorothioate ODN (S-ODN) (0.5 μM), or E. coli or calf thymus DNA (50 μg/ml) at. . . IgM production). Concentrations of stimulants were chosen based on preliminary studies with titrations. In some cases, cells were treated with **CpG** O-ODN along with various concentrations (1-10 μg/ml) of neutralizing rat IgG1 antibody against murine IL-6 (hybridoma MP5-20F3) or control rat. . .

DETD . . . injected intravenously (iv) with PBS, calf thymus DNA (200 μg/100 μl PBS/mouse), E. coli DNA (200 μg/100 μl PBS/mouse), or **CpG** or non-**CpG** S-ODN (200 μg/100 μl PBS/mouse). Mice (two/group) were bled by retroorbital puncture and sacrificed by cervical dislocation at various time . . .

DETD Cell Proliferation assay. DBA/2 mice spleen B cells (5×10^{4} cells/100 μl/well) were treated with media, **CpG** or non-**CpG** S-ODN (0.5 μM) or O-ODN (20 μM) for 24 hr at 37° C. Cells were pulsed for the last four. . .

DETD . . . a receptor-dependent mechanism. J. Clin. Invest. 93:944) at 250 mV and 960 μF. Cells were stimulated with various concentrations or **CpG** or non-**CpG** ODN after electroporation. Chloramphenicol acetyltransferase (CAT) activity was measured by a solution assay (Seed, B. and J. Y. Sheen (1988). . .

DETD Oligodeoxynucleotide Modifications Determine the Magnitude of B Cell Stimulation by **CpG** Motifs

DETD . . . central linkages are phosphodiester, but the two 5' and five 3' linkages are methylphosphonate modified. The ODN sequences studied (with **CpG** dinucleotides indicated by underlining) include:

DETD These sequences are representative of literally hundreds of **CpG** and non-**CpG** ODN that have been tested in the course of these studies.

DETD . . . (1990) J. Immunol 145:1039; and Ballas, Z. K. and W. Rasmussen (1993) J. Immunol, 150:17), with medium alone or with **CpG** or non-**CpG** ODN at the indicated concentrations, or with E.coli or calf thymus (50 μg/ml) at 37° C. for 24 hr. All. . .

DETD . . . immunized mice were then treated with oligonucleotides (30 μg in 200 μl saline by i.p.injection), which either contained an unmethylated **CpG** motif (i.e., **TCCATGACGTTCCTGACGTT**; SEQ ID NO.10) or did not (i.e., control, TCCATGAGCTTCCTGAGTCT; SEQ ID NO.8). Soluble SEA (10 μg in 25 μl of. . .

DETD . . . inflammatory cells are present in the lungs. However, when the mice are initially given a nucleic acid containing an unmethylated **CpG** motif along with the eggs, the inflammatory cells in the lung are not increased by subsequent inhalation of the egg. . .

DETD . . . inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a **CpG** oligo along

with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes. . .

DETD FIG. 14 shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can actually redirect the cytokine response of the lung to production of Il-12, indicating a Th1 type of immune. . .

DETD FIG. 15 shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune. .

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DETD **CpG** Oligonucleotides Induce Human PBMC to Secrete Cytokines

DETD . . . standard centrifugation over ficoll hypaque. Cells (5×10^5 /ml) were cultured in 10% autologous serum in 96 well microtiter plates with **CpG** or control oligodeoxynucleotides (24 μg/ml for phosphodiester oligonucleotides; 6 μ/ml for nuclease resistant phosphorothioate oligonucleotides) for 4 hr in the. . .

. . . Il-6 in a subject comprising administering to the subject an effective amount to induce Il-6 in the subject of an **immunostimulatory** nucleic acid, having a sequence comprising: 5'X_1 X_2 CGX_3 X3' wherein C is unmethylated, wherein X_1, X_2 and X_3, X_4. . .

. . . 1, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, GpT, GpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

11. The method of claim 1, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

12. The method of claim 1, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and. .

.

13. The method of claim 1, wherein the **immunostimulatory** nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCCATGTCGCTCCTGATGCT (SEQ ID NO:37); TCCATAACGTTCCTGATGCT. . .

14. A method of stimulating natural killer cell lytic activity comprising exposing a natural killer cell to an **immunostimulatory** nucleic acid to stimulate natural killer cell lytic activity, the **immunostimulatory** nucleic acid having a sequence comprising: 5'X_1 X_2 CGX_3 X3' wherein C is unmethylated, wherein X_1 X_2 and X_3 X_4. . .

. . . 14, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

23. The method of claim 14, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

24. The method of claim 15, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and.

. .

25. The method of claim 14, wherein the **immunostimulatory** nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCGTCGTTGTCGTTGTCGTT (SEQ ID NO:47); TCCATGACGGTCCTGATGCT. . .

. . . to a subject having an immune system deficiency an effective amount to induce interferon-gamma production in the subject of an **immunostimulatory** nucleic acid, having a sequence comprising: 5'X_1 X_2 CGX_3 X_4 3' wherein C is unmethylated, wherein X_1 X_2 and X_3. . .

. . . 26, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

35. The method of claim 26, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

36. The method of claim 26, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and.

. .

. . . Il-12 in a subject comprising: administering to the subject an effective amount to induce Il-12 in the subject, of an **immunostimulatory** nucleic acid having a sequence comprising: 5'X_1 X_2 CGX_3 X3' wherein C is unmethylated, wherein X_1, X_2, X_3, and X_4. . .

39. The method of claim 37, wherein the **immunostimulatory** nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCCATGTCGCTCCTGATGCT (SEQ ID NO:37); TCCATGACGATCCTGATGCT. . .

. . . 37, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

48. The method of claim 37, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

49. The method of claim 37, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and. . .

L15 ANSWER 8 OF 12 USPATFULL on STN

2001:55947 Methods and products for stimulating the immune system using immunotherapeutic oligonucleotides and cytokines.

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US 6218371 B1 20010417

APPLICATION: US 1999-286098 19990402 (9)

PRIORITY: US 1998-80729P 19980403 (60)

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A method for stimulating an immune response in a subject, comprising: administering to a subject exposed to an antigen an effective amount for inducing a synergistic antigen specific immune response of an immunopotentiating cytokine selected from the group consisting of GM-CSF, IL-2, IL-4 and IFN-γ, and an **immunostimulatory CpG** oligonucleotide having a sequence including at least the following formula: 5' X_1 CGX_2 3' wherein the oligonucleotide includes at least 8 nucleotides wherein C is unmethylated and wherein X_1 and X_2 are nucleotides, wherein the cytokine is a peptide, whereby an antigen is optionally additionally administered, and wherein the antigen and the **CpG** oligonucleotide are not conjugated.

2. A The method of claim 1, wherein the immunopotentiating cytokine is an antigen-cytokine fusion protein.

3. The method of claim 2, wherein the antigen-cytokine fusion protein is an antigen-GM-CSF fusion protein.

4. The method of claim 1, wherein the antigen is selected from the group consisting of a tumor antigen, a microbial antigen, and an allergen.

5. The method of claim 4, wherein the antigen is a tumor antigen.

6. The method of claim 1, wherein the antigen is administered to the subject in conjunction with the **immunostimulatory CpG** oligonucleotide and the immunopotentiating cytokine.

7. The method of claim 1, wherein the subject is passively exposed to the antigen.

8. The method of claim 1, wherein the subject has a neoplastic disorder.

9. The method of claim 1, wherein the subject has a viral infection.

10. The method of claim 1, wherein the subject is a non-human animal.

11. The method of claim 10, wherein the non-human animal is a vertebrate animal selected from the group consisting of a dog, a cat, a horse, a cow, a pig, a sheep, a goat, a chicken, and a primate.

12. A composition, comprising: an effective amount for synergistically activating a dendritic cell of an **immunostimulatory CpG**

oligonucleotide having a sequence including at least the following formula: 5' X_1 CGX_2 3' wherein the oligonucleotide includes at least 8 nucleotides wherein C is unmethylated and wherein X_1 and X_2 are nucleotides; and a cytokine selected from the group consisting of GM-CSF, IL-2, IL-4 and IFN-γ, wherein the cytokine is a peptide.

13. The composition of claim 12, wherein the cytokine is GM-CSF.

14. The composition of claim 12, further comprising an antigen and wherein the antigen and the **CpG** oligonucleotide are not conjugated.

15. The composition of claim 14, wherein the antigen is selected from the group consisting of a cancer antigen, a microbial antigen, and an allergen.

16. A method for activating a dendritic cell, comprising: contacting a dendritic cell exposed to an antigen with an effective amount for synergistically activating a dendritic cell of an immunopotentiating cytokine selected from the group consisting of GM-CSF, IL-2, IL-4 and IFN-γ, and an **immunostimulatory CpG** oligonucleotide having a sequence including at least the following formula: 5' X_1 CGX_2 3' wherein the oligonucleotide includes at least 8 nucleotides wherein C is unmethylated and wherein X_1 and X_2 are nucleotides, wherein the cytokine is a peptide, whereby an antigen is optionally additionally administered, and wherein the antigen and the **CpG** oligonucleotide are not conjugated.

17. The method of claim 16, wherein the antigen is a tumor antigen.

18. A method for treating a subject having a neoplastic disorder, comprising: administering to the tumor of a subject having a neoplastic disorder an immunopotentiating cytokine selected from the group consisting of GM-CSF, IL-2, IL-4 and IFN-γ, and an **immunostimulatory CpG** oligonucleotide having a sequence including at least the following formula: 5' X_1 CGX_2 3' wherein the oligonucleotide includes at least 8 nucleotides wherein C is unmethylated and wherein X_1 and X_2 are nucleotides, in an amount effective for synergistically increasing survival time of the subject with respect to a subject administered the **immunostimulatory CpG** oligonucleotide or the immunopotentiating cytokine alone, where the cytokine is a peptide.

19. The method of claim 18, wherein the tumor is selected from the group consisting of a lymphoma and a tumor of the brain, lung, ovary, breast, prostate, colon, and skin.

20. The method of claim 18, wherein the **immunostimulatory CpG** oligonucleotide and the immunopotentiating cytokine are injected directly into the tumor.

21. The method of claim 18, wherein the subject is a non-human animal.

22. The method of claim 21, wherein the non-human animal is a vertebrate animal selected from the group consisting of a dog, a cat, a horse, a cow, a pig, a sheep, a goat, a chicken, and a primate.

23. The method of claim 22, wherein the tumor is selected from the group consisting of lymphoma an a tumor of the brain, lung, ovary, breast, prostate, colon, and skin.

AI **US 1999-286098 19990402 (9)**

AB The present invention relates to synergistic combinations of **immunostimulatory CpG** oligonucleotides and immunopotentiating cytokines. In particular, the invention relates to methods of stimulating an immune response using the synergistic combination. . .

SUMM The present invention relates to synergistic combinations of **immunostimulatory CpG** oligonucleotides and immunopotentiating cytokines. In particular, the invention relates to methods of stimulating an immune response using the synergistic combination. . .

SUMM . . . has potent immunostimulator effects, and vertebrate DNA, which does not, is that bacterial DNA contains a higher frequency of unmethylated **CpG** dinucleotides than does vertebrate DNA. Select synthetic oligodeoxynucleotides (ODN) containing unmethylated **CpG** motifs (**CpG** ODN) have been shown to have an immunologic effects and can induce activation of B cells, NK cells and antigen-presenting. . .

SUMM The present invention relates to methods and products for inducing a synergistic immune response using a combination of a **CpG** oligonucleotide and a cytokine. In one aspect the invention is a method

for stimulating an immune response in a subject.. . . to an antigen an effective amount for inducing a synergistic antigen specific immune response of an immunopotentiating cytokine and an **immunostimulatory CpG** oligonucleotide having a sequence including at least the following formula:

SUMM In some embodiments the antigen is administered to the subject in conjunction with the **immunostimulatory CpG** oligonucleotide and the immunopotentiating cytokine. In other embodiments the subject is passively exposed to the antigen.

SUMM In other aspects the invention is a composition of an effective amount for synergistically activating a dendritic cell of an **immunostimulatory CpG** oligonucleotide having a sequence including at least the following formula:

SUMM . . . exposed to an antigen with an effective amount for synergistically activating a dendritic cell of an immunopotentiating cytokine and an **immunostimulatory CpG** oligonucleotide having a sequence including at least the following formula:

SUMM . . . includes the step of administering to the tumor of a subject having a neoplastic disorder an immunopotentiating cytokine and an **immunostimulatory CpG** oligonucleotide having a sequence including at least the following formula:

SUMM . . . nucleotides, in an amount effective for synergistically increasing survival time of the subject with respect to a subject administered the **immunostimulatory CpG** oligonucleotide or the immunopotentiating cytokine alone.

SUMM . . . group consisting of a tumor of the brain, lung, ovary, breast, prostate, colon, skin, and blood. In one embodiment the **immunostimulatory CpG** oligonucleotide and the immunopotentiating cytokine are injected directly into the tumor.

SUMM . . . of the invention. The method involves the step of administering to a subject an antigen, an immunopotentiating cytokine and an **immunostimulatory CpG** oligonucleotide having a sequence including at least the following formula:

DRWD FIG. 1 is a graph showing the production of anti-Id IgG following immunization using a combination of **CpG** ODN and soluble GM-CSF. Mice were immunized with 50 μg of Id-KLH as a single subcutaneous dose mixed in aqueous solution with GM-CSF, **CpG** ODN or both. Blood was obtained weekly, and serum was evaluated for the presence of anti-Id IgG by ELISA. Normal. . .

DRWD FIG. 2 is a graph showing that immunization using a combination of Id/GM-CSF fusion protein and **CpG** ODN enhances production of antigen-specific IgG. Mice were immunized with 50 μg of Id/GM-CSF as a single subcutaneous dose with or without **CpG** ODN. Blood was obtained weekly, and serum was evaluated for the presence of anti-Id IgG by ELISA. Normal mouse serum. . .

DRWD FIG. 3 is a graph showing that immunization using repeated immunizations with a combination of Id/GM-CSF fusion protein and **CpG** ODN induces high levels of antigen-specific IgG. Mice were immunized with 50 μg of Id/GM-CSF as a subcutaneous dose with or without **CpG** ODN on week 0 and again on week 2. Blood was obtained weekly, and serum was evaluated for the presence. . .

DRWD FIG. 4 is a bar graph showing that **CpG** ODN enhances production of antigen specific antibody of IgG_{2a} isotype. Mice were immunized with a single dose using various combinations of Id-KLH, GM-CSF, Id/GM-CSF fusion protein, and **CpG** ODN. Serum was obtained 4 weeks after a single immunization. Anti-d IgG_1 and IgG_{2a} was determined by ELISA. Three mice. . .

DRWD FIG. 5 is a survival curve showing that **CpG** ODN enhances the protective effect of Id/GM-CSF protection against tumor growth. Mice were immunized with a single injection of Id/GM-CSF and/or **CpG** ODN and challenged with tumor 3 days later. Survival was followed for 100 days. All mice that were alive after. . .

DRWD . . . of MHC class I, MHC class II, CD80, and CD86 after pulsing of bone marrow-derived dendritic cells with Id/GM-CSF and/or **CpG** ODN.

DRWD FIG. 7 is a bar graph illustrating that **CpG** ODN enhances production IL-12 by dendritic cells pulsed with Id-KLH or Id/GM-CSF. Bone marrow derived dendritic cells were pulsed with antigen with and without **CpG** ODN for 18 hours, and production of IL-12 and IL-6 determined by ELISA. **CpG** ODN markedly enhanced production of IL-12 by dendritic cells, particularly those pulsed with the Id/GM-CSF fusion protein.

DRWD FIG. 8 shows FACS charts demonstrating that ICAM-1 and MHC II expression of dendritic cells in response to GM-CSF and **CpG**. Dendritic precursor cells were incubated for 48 hours in the presence of GM-CSF (800 U/ml) and 2006 (**CpG** phosphorothioate; 6 μg/ml). Expression of ICAM-1 (CD54) and MHC II was examined by flow cytometry (2500 viable cells are counted. . .

DRWD FIG. 9 is several graphs depicting induction of co-stimulatory molecule expression on dendritic cells by **CpG**. Dendritic precursor cells were incubated for 48 hours in the presence of GM-CSF (800 U/ml) and

oligonucleotides (2006: **CpG** phosphorothioate, 6 μg/ml) as indicated. Expression of CD54 (ICAM-1) (panel A), CD86 (B7-2) (panel B) and CD40 (panel C) was. . .

DETD . . . to an antigen an effective amount for inducing a synergistic antigen specific immune response of an immunopotentiating cytokine and an **immunostimulatory CpG** oligonucleotide.

DETD The finding is based on the discovery that when an **immunostimulatory CpG** oligonucleotide is administered to a subject in combination with an immunopotentiating cytokine the resultant immune response is synergistic. Both **CpG** oligonucleotides and immunopotentiating cytokines have the ability to produce immune responses on their own when administered to a subject. When. . . combination of the two is administered together, however, the quantity and type of immune response shifts. For instance, when the **CpG** oligonucleotide and immunopotentiating cytokine are administered in conjunction with an antigen using repeat immunizations, as shown in FIG. 3, a synergistic induction in antigen specific IgG is observed. Additionally, when **CpG** and GM-CSF are administered together an antibody response develops that includes both IgG2a (indicative of a Th1 immune response) and. . .

DETD Amazingly, the combination of a **CpG** oligonucleotide and immunopotentiating cytokine has a dramatic effect on the survival rate of animals injected with a tumor, even when. . . injected with a tumor and not provided with any subsequent tumor therapy the survival rate was 0%. Mice treated with **CpG** oligonucleotide alone or GM-CSF and antigen had survival rates of 0 and 30% respectively. The combination of **CpG** oligonucleotide and GM-CSF produced a dramatic survival rate of 70%. This finding has serious implications for the treatment of established. . .

DETD . . . to an antigen an effective amount for inducing a synergistic antigen specific immune response of an immunopotentiating cytokine and an **immunostimulatory CpG** oligonucleotide. The **immunostimulatory CpG** oligonucleotide has a sequence including at least the following formula:

DETD **CpG** oligonucleotide can be useful in activating B cells, NK cells, and antigen-presenting cells, such as monocytes and macrophages. **CpG** oligonucleotide enhances antibody dependent cellular cytotoxicity and can be used as an adjuvant in conjunction with tumor antigen to protect. . . Weiner, G. J., et al., Proc. Natl. Acad. Sci. USA 94:10833-10837, 1997). This invention is based on the finding that **CpG** oligonucleotide and an immunopotentiating cytokine act synergistically in order to produce an immune response against a tumor, such that the effect of **CpG** oligonucleotide and the immunopotentiating agent is greater than the sum of the individual effects of either **CPG** oligonucleotide or the immunopotentiating agent.

DETD In the method of the invention, **CpG** oligonucleotide are used with an immunopotentiating cytokine. "Immunopotentiating cytokines" are those molecules and compounds which stimulate the humoral and/or cellular. . .

DETD . . . An infectious disease, as used herein, is a disease arising from the presence of a foreign microorganism in the body. **CpG** and immunopotentiating cytokine are used to stimulate an antigen specific immune response which can activate a T or B cell. . .

DETD Allergies are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated **CpG** oligonucleotides are predominantly of a class called "Th1" which is most marked by a cellular immune response and is associated. . . by Th2 type immune responses and autoimmune diseases by Th1 immune response. Based on the ability of the combination of **CpG** oligonucleotides and immunopotentiating cytokine to shift the immune response in a subject from a Th2 (which is associated with production. . . in response to GM-CSF alone) to a Th1 response (which is protective against allergic reactions), an effective dose of a **CpG** oligonucleotide and immunopotentiating cytokine can be administered to a subject to treat or prevent an allergy.

DETD **CpG** oligonucleotides combined with immunopotentiating cytokines may also have significant therapeutic utility in the treatment of asthma. Th2 cytokines, especially IL-4. . .

DETD As described in co-pending patent application U.S. Ser. No. 08/960,774, oligonucleotides containing an unmethylated **CpG** motif (i.e. **TCCATGACGTTCCTGACGTT**; SEQ IN NO: 93), but not a control oligonucleotide (TCCATGAGCTTCCTGAGTCT; SEQ ID NO: 103) prevented the development of an inflammatory. . .

DETD Thus the present invention contemplates the use of **CpG** oligonucleotides and immunopotentiating cytokines to induce an antigen specific immune response in human and non-human animals. As discussed above, antigens. . .

DETD In addition to the use of the combination of **CpG** oligonucleotides and immunopotentiating cytokines to induce an antigen specific immune response in humans, the methods of the preferred embodiments are. . .

DETD . . . birds are housed in closed quarters, leading to the rapid spread of disease. Thus, it is desirable to administer the **CpG** oligonucleotide and the immunopotentiating cytokine of the invention to birds to enhance an antigen-specific immune response when antigen is present.

DETD . . . may be administered subcutaneously, by spray, orally, intraocularly, intratracheally, nasally, in ovo or by other methods described herein. Thus, the **CpG** oligonucleotide and immunopotentiating cytokine of the invention can be administered to birds and other non-human vertebrates using routine vaccination schedules. . .

DETD . . . intranasal, intratracheal, or subcutaneous administration. The antigen can be administered systemically or locally. Methods for administering the antigen and the **CpG** and immunopotentiating cytokine are described in more detail below. A subject is passively exposed to an antigen if an antigen. . . a foreign antigen on its surface. When a subject is passively exposed to an antigen it is preferred that the **CpG** oligonucleotide is an oligonucleotide of 8-100 nucleotides in length and/or has a phosphate modified backbone.

DETD The methods in which a subject is passively exposed to an antigen can be particularly dependent on timing of **CpG** oligonucleotide and immunopotentiating cytokine administration. For instance, in a subject at risk of developing a cancer or an infectious disease or an allergic or asthmatic response, the subject may be administered the **CpG** oligonucleotide and immunopotentiating cytokine on a regular basis when that risk is greatest, i.e., during allergy season or after exposure to a cancer causing agent. Additionally the **CpG** oligonucleotide and immunopotentiating cytokine may be administered to travelers before they travel to foreign lands where they are at risk of exposure to infectious agents. Likewise the **CpG** oligonucleotide and immunopotentiating cytokine may be administered to soldiers or civilians at risk of exposure to biowarfare to induce a. . .

DETD . . . cancer can also be treated according to the methods of the invention, by passive or active exposure to antigen following **CpG** and immunopotentiating cytokine. A subject at risk of developing a cancer is one who is who has a high probability. . . such as tobacco, asbestos, or other chemical toxins. When a subject at risk of developing a cancer is treated with **CpG** and immunopotentiating cytokine on a regular basis, such as monthly, the subject will be able to recognize and produce an. . .

DETD . . . antigen must be expressed in vivo. In these embodiments of the invention the nucleic acids molecule may also include a **CpG** dinucleotide within the sequence of the nucleic acid. But in this case the nucleic acid molecule does not take the place of the **CpG** oligonucleotide. The antigen must be administered in conjunction with a **CpG** oligonucleotide that is separate from the nucleic acid molecule. The nucleic acid encoding the antigen is operatively linked to a. . .

DETD Thus, the invention contemplates scheduled administration of **CpG** oligonucleotides and immunopotentiating cytokine. The oligonucleotides may be administered to a subject on a weekly or monthly basis. When a subject is at risk of exposure to an antigen or antigens the **CpG** and immunopotentiating cytokine may be administered on a regular basis to recognize the antigen immediately upon exposure and produce an. . .

DETD The **CpG** oligonucleotides of the invention are nucleic acid molecules which contain an unmethylated cytosine-guanine dinucleotide sequence (i.e. "**CpG** DNA" or DNA containing a 5' cytosine followed by 3' guanosine and linked by a phosphate bond) and activate the immune system. The **CpG** oligonucleotides can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have increased immune. . .

DETD . . . from existing nucleic acid sources (e.g. genomic or cDNA), but are preferably synthetic (e.g. produced by oligonucleotide synthesis). The entire **CpG** "oligonucleotide can be unmethylated or portions may be unmethylated but at least the C of the 5' CG 3' must. . .

DETD In one preferred embodiment the invention provides a **CpG** oligonucleotide represented by at least the formula:

DETD In another embodiment the invention provides an isolated **CpG** oligonucleotide represented by at least the formula:

DETD . . . acid do not contain a CCGG quadmer or more than one CCG or CGG trimer. In another preferred embodiment the **CpG** oligonucleotide has the sequence 5'TCN_1 TX_1 X_2 CGX_3 X_4 3'.

DETD Preferably the **CpG** oligonucleotides of the invention include X_1 X_2 selected from the group consisting of GpT, GpG, GpA and ApA and X_3 X_4 is selected from the group consisting of TpT, CpT and GpT. For facilitating uptake into cells, **CpG** containing oligonucleotides are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size. . . than 8 nucleotides (even many kb long) are capable of inducing an immune response according to the invention if sufficient **immunostimulatory** motifs are present, since larger nucleic acids are degraded into oligonucleotides inside of cells.

Preferred synthetic oligonucleotides do not include. . .

DETD Preferably the **CpG** oligonucleotide is in the range of between 8 and 100 and more preferably between 8 and 30 nucleotides in size. Alternatively, **CpG** oligonucleotides can be produced on a large scale in plasmids and degraded into oligonucleotides.

DETD The **CpG** oligonucleotide and immunopotentiating cytokine may be directly administered to the subject or may be administered in conjunction with a nucleic. . .

DETD . . . capable of forming the usual Watson-Crick base pairs. In vivo, such sequences may form double-stranded structures. In one embodiment the **CpG** oligonucleotide contains a palindromic sequence. A palindromic sequence used in this context refers to a palindrome in which the **CpG** is part of the palindrome, and preferably is the center of the palindrome. In another embodiment the **CpG** oligonucleotide is free of a palindrome. A **CpG** oligonucleotide that is free of a palindrome is one in which the **CpG** dinucleotide is not part of a palindrome. Such an oligonucleotide may include a palindrome in which the **CpG** is not part of the palindrome.

DETD . . . in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated **CpG** oligonucleotides that are tens to hundreds of kbs long are relatively resistant to in vivo degradation. For shorter **CpG** oligonucleotides, secondary structure can stabilize and increase their effect. For example, if the 3' end of an oligonucleotide has self-complementarity. . .

DETD . . . invention have a modified backbone. It has been demonstrated that modification of the oligonucleotide backbone provides enhanced activity of the **CpG** oligonucleotides when administered in vivo. **CpG** constructs, including at least two phosphorothioate linkages at the 5' end of the oligonucleotide in multiple phosphorothioate linkages at the. . .

DETD Both phosphorothioate and phosphodiester oligonucleotides containing **CpG** motifs are active in APCs such as dendritic cells. However, based on the concentration needed to induce **CpG** specific effects, the nuclease resistant phosphorothioate backbone **CpG** oligonucleotides are more potent (2 μg/ml for the phosphorothioate vs. a total of 90 μg/ml for phosphodiester).

DETD . . . 08/960,774, filed on Oct. 30, 1996 and Oct. 30, 1997 respectively. Exemplary sequences include but are not limited to those **immunostimulatory** sequences shown in Table 1 as well as TCCATGTCGCTCCTGATGCT (SEQ ID NO: 47), TCCATGTCGTTCCTGATGCT (SEQ ID NO: 48), TCGTCGTTTTGTCGTTTTGTCGTT (SEQ. . . TCGTCGCTGTCTGCCCTTCTT (SEQ ID NO:82), TCGTCGCTGTTGTCGTTTCTT (SEQ ID NO:83), TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:90), TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO:91), TGTCGTTGTCGTTGTCGTT (SEQ ID NO:96), **TCCATGACGTTCCTGACGTT** (SEQ ID NO:100), GTCG(T/C)T (SEQ ID NO:101) and TGTCG(T/C)T (SEQ ID NO: 102).

DETD The stimulation index of a particular **immunostimulatory CpG** DNA can be tested in various immune cell assays. Preferably, the stimulation index of the **immunostimulatory CpG** DNA with regard to B cell proliferation is at least about 5, preferably at least about 10, more preferably at. . . treat an immune system deficiency by stimulating a cell-mediated (local) immune response in a subject, it is important that the **immunostimulatory CpG** DNA be capable of effectively inducing cytokine secretion by APCs such as dendritic cells.

DETD Preferred **immunostimulatory CpG** nucleic acids should effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml. . . IL-6, 200 pg/ml IL-12, depending on the therapeutic indication, as determined by the assays described in the Examples. Other preferred **immunostimulatory CpG** DNAs should effect at least about 10%, more preferably at least about 15% and most preferably at least about 20%. . . least about 40% 2C11 cell specific lysis. When administered in conjunction with an immunopotentiating cytokine the amounts of both the **CpG** oligonucleotide and the cytokine required to produce a desired immune response will be less.

DETD Preferably, the stimulation index of the **CpG** oligonucleotide with regard to B cell proliferation is at least about 5, preferably at least about 10, more preferably at. . . on Oct. 30, 1996 and Oct. 30, 1997 respectively. For use in vivo, for example, it is important that the **CpG** oligonucleotide and cytokine be capable of effectively inducing activation of APC's such as dendritic cells. Oligonucleotides which can accomplish this. . .

DETD **CpG** oligonucleotides and immunopotentiating cytokines can be administered to a subject alone prior to the administration of an antigen. The oligonucleotides. . . different from the first antigen may then be administered to the subject at some time point after the administration of **CpG** and immunopotentiating cytokine in the presence or absence of additional **CpG** and cytokine. The term "in conjunction with" refers to the administration of the **CpG** oligonucleotide and immunopotentiating cytokine slightly before or slightly after or at the

same time as the antigen. The terms slightly before and slightly after refer to a time period of 24 hours and preferably 12 hours. The **CpG** and cytokine are administered in conjunction with one another and thus may also be administered together or separately.

DETD When the **CpG** oligonucleotide and immunopotentiating cytokine are administered in conjunction with a first antigen the first antigen will determine the specificity of the immediate immune response. The **CpG** oligonucleotide and immunopotentiating cytokine act as an effective "danger signal" and cause the immune system to respond vigorously to new antigens in the area. This mode of action presumably results primarily from the stimulatory local effects of **CpG** oligonucleotide and immunopotentiating cytokine on dendritic cells and other "professional" antigen presenting cells, as well as from the co-stimulatory effects on B cells. This effect occurs immediately upon the administration of the **CpG** oligonucleotide.

DETD For use in therapy, an effective amount of an appropriate **CpG** oligonucleotide and immunopotentiating cytokine alone or formulated as a nucleic acid/cytokine delivery complex can be administered to a subject by. . .

DETD The term "effective amount" of a **CpG** oligonucleotide refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of an oligonucleotide containing at least one unmethylated **CpG** for treating an immune system deficiency could be that amount necessary to cause activation of the immune system, resulting in. . . an immune response against a specific antigen that is greater than the sum of the individual effects of either the **CpG** or the cytokine alone.

DETD . . . amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular **CpG** oligonucleotide/cytokine being administered (e.g. the number of unmethylated **CpG** motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or. . .

DETD Another use for **CpG** oligonucleotide in combination with an immunopotentiating cytokine is the production of a contraceptive method for use in a subject. In. . . and the expression of fas ligand both prevent an immune response against the cells in the testes and ovaries. The **CpG** oligonucleotide used in conjunction with an immunopotentiating cytokine can be used to eliminate or substantially reduce the cells in the testes and the ovaries by breaking the immune privilege of these cells, thereby providing a contraceptive means. **CpG** oligonucleotide can be used in conjunction with an immunopotentiating cytokine to break the immune privilege of the cells of the. . .

DETD The method is accomplished by administering to a subject an antigen, an immunopotentiating cytokine and an **immunostimulatory CpG** oligonucleotide, wherein the antigen is an antigen selected from the group consisting of a gonadal cell antigen and an antigen. . .

DETD The **CpG** oligonucleotides are used in one aspect of the invention to induce activation of immune cells and preferably APCs. An APC. . .

DETD . . . of pattern recognition receptors which detect microbial molecules like LPS in their local environment. The combination of immunopotentiating cytokine and **CpG** oligonucleotide showed induction of Th1 specific antibody when immunopotentiating cytokine alone only produced Th2 specific antibody. Since dendritic cells form the link between the innate and the acquired immune system the ability to activate dendritic cells with **CpG** and immunopotentiating cytokine supports the use of combination **CpG**-immunopotentiating cytokine based strategies for immunotherapy against disorders such as cancer and allergic or infectious diseases. The combination of **CpG** and immunopotentiating cytokine shows synergistic activation of dendritic cells.

DETD . . . ex vivo and in vivo purposes. It was demonstrated according to the invention that the combination of immunopotentiating cytokine and **CpG** oligonucleotide is a potent activator of dendritic cells. Dendritic cells are believed to be essential for the initiation of primary immune responses in immune cells in vivo. It was discovered, according to the invention, that **CpG** oligonucleotides and immunopotentiating cytokine were capable of activating dendritic cells to initiate primary immune responses in T cells, similar to an adjuvant. It was also discovered that when the combination of the **CpG** oligonucleotide and immunopotentiating cytokine is used to activate dendritic cells the production of predominantly IgG2a and less IgG1 is induced,. . . its propensity to augment the development of Th1 immune responses in vivo. These findings demonstrate the potent adjuvant activity of **CpG** and provide the basis for the use of **CpG** oligonucleotides as immunotherapeutics in the treatment of disorders such as cancer, infectious diseases, and allergy. In one aspect, the invention. . . exposed to an antigen with an effective amount for synergistically activating a dendritic cell of an immunopotentiating cytokine and an **immunostimulatory CpG** oligonucleotide.

DETD One specific use for the combination of **CpG** oligonucleotide and immunopotentiating cytokine of the invention is to activate dendritic cells for the purpose of enhancing a specific immune. . . specific cancer or other type of antigen, the dendritic cells may be exposed to the antigen in addition to the **CpG** and immunopotentiating cytokine. In other cases the dendritic cell may have already been exposed to antigen but may not be. . . contact or exposure in the body and then the dendritic cell is returned to the body followed by administration of **CpG** directly to the subject, either systemically or locally. Activation will dramatically increase antigen processing. The activated dendritic cell then presents. . . the invention may be performed by routine ex vivo manipulation steps known in the art, but with the use of **CpG** and immunopotentiating cytokine as the activator.

DETD The dendritic cells may also be contacted with **CpG** and immunopotentiating cytokine using in vivo methods. In order to accomplish this, **CpG** and immunopotentiating cytokine are administered directly to a subject in need of immunotherapy. The **CpG** and immunopotentiating cytokine may be administered in combination with an antigen or may be administered alone. In some embodiments, it is preferred that the **CpG** and immunopotentiating cytokine be administered in the local region of the tumor, which can be accomplished in any way known. . .

DETD . . . to the invention may be isolated from any source as long as the cell is capable of being activated by **CpG** and cytokine to produce an active antigen expressing dendritic cell. Several in vivo sources of immature dendritic cells may be. . . marrow dendritic cells and peripheral blood dendritic cells are both excellent sources of immature dendritic cells that are activated by **CpG** and cytokine. Other sources may easily be determined by those of skill in the art without requiring undue experimentation, by for instance, isolating a primary source of dendritic cells and testing activation by **CpG** in vitro. The invention also encompasses the use of any immature dendritic cells maintained in culture as a cell line as long as the cell is capable of being activated by **CpG** and cytokine. Such cell types may be routinely identified using standard assays known in the art.

DETD Peripheral blood dendritic cells isolated by immunomagnetic cell sorting, which are activated by **CpG** and cytokine, represent a more physiologic cell population of dendritic cells than monocyte derived dendritic cells. Immature dendritic cells comprise. . . flow cytometry. Freshly isolated dendritic cells cultured in the absence of GM-CSF rapidly undergo apoptosis. Strikingly, in the presence of **CpG** oligonucleotides without addition of GM-CSF, both cell survival and differentiation is markedly improved compared to GM-CSF. In the presence of **CpG**, dendritic cells form cell clusters which when examined by ultrastructural techniques such as electron microscopy revealed characteristic dense multilamellar intracytoplasmic. . . and had only minor cellular processes. In addition to promoting survival and differentiation of dendritic cells, a single addition of **CpG** oligonucleotide led to activation as represented by upregulation of the co-stimulatory molecules ICAM-1 (CD54), B7-2 (CD86) and CD40. The combination of **CpG** oligonucleotide and GM-CSF enhanced the expression of CD86 and CD40 synergistically, proving that activation is not due to **CpG**-induced GM-CSF.

DETD Method for Making **Immunostimulatory** Nucleic Acids

DETD . . . described (Uhlmann, E. and Peyman, A., 1990, Chem. Rev. 90:544; Goodchild, J., 1990, Bioconjugate Chem. 1:165). 2'-O-methyl nucleic acids with **CpG** motifs also cause immune activation, as do ethoxy-modified **CpG** nucleic acids. In fact, no backbone modifications have been found that completely abolish the **CpG** effect, although it is greatly reduced by replacing the C with a 5-methyl C.

DETD The compositions of the invention, including activated dendritic cells, isolated **CpG** nucleic acid molecules, cytokines, and mixtures thereof are administered in pharmaceutically acceptable compositions. When administered, the compositions of the invention. . . alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts. As used herein, a composition of a **CpG** oligonucleotide and/or an immunopotentiating cytokine means the compounds described above as well as salts thereof.

DETD Two phosphorothioate **CpG** oligonucleotides were purchased commercially and produced under GMP conditions (Oligos Etc., Wilsonville, Oreg.). Both oligonucleotide sequences had similar effects in all assays. **CpG** oligonucleotide 1758 was used unless stated otherwise. Oligonucleotide 1758 had the sequence

DETD **TCCATGACGTTCCTGACGTT** (SEQ ID NO:3)

DETD Both **CpG** oligonucleotide were unmethylated. No detectable endotoxin was present in either **CpG** oligonucleotide by LAL assay. Prior studies demonstrated non-**immunostimulatory** oligonucleotide had little adjuvant effect (Weiner, G. J., et al., Proc. Natl. Acad. Sci. USA 94:10833-10837, 1997), therefore non-**immunostimulatory** oligonucleotide

were not included in the current studies. Murine GM-CSF for in vitro production of dendritic cells it was purchased. . .

DETD . . . for 18 hours in a total volume of 200 μl with antigen at a final concentration of 100 μg/ml and **CpG** oligonucleotide at a final concentration of 50 μg/ml. For measurement of cytokine levels, all samples were run in quadruplicate. Supernatant. . .

DETD **CpG** Oligonucleotide Enhances Development of an Antibody Response to Id-KLH Immunization When Using GM-CSF as an Adjuvant

DETD **CpG** oligonucleotide is known to induce production by APCs of a number of cytokines including GM-CSF (Krieg, A. M., Trends in Microbiology 4:73-6, 1996). In order to determine if the addition of **CpG** oligonucleotide to GM-CSF would further enhance the immune response mice were immunized with a single subcutaneous injection of 50 μg of Id-KLH in PBS mixed in aqueous solution with 50 μg of **CpG** oligonucleotide, 10 μg of GM-CSF, or a combination of **CpG** oligonucleotide and GM-CSF. Serum was obtained weekly and evaluated by ELISA for the presence of antigen-specific IgG (anti-Id IgG). As illustrated in FIG. 1, mice immunized using both **CpG** oligonucleotide and GM-CSF developed the highest levels of anti-Id IgG. The effect of these two adjuvants appeared to be additive.

DETD The combination of GM-CSF and **CpG** oligonucleotide could therefore enhance a number of different steps in the induction of the immune response with GM-CSF increasing antigen uptake while **CpG** oligonucleotide enhances the downstream response including production of cytokines involved in effector cell activation. In addition, **CpG** oligonucleotide contributes by synergistically promoting B-cell activation through the antigen receptor, and so preferentially activating antigen-specific B-cells (Krieg, A. M., et al., Nature 374:546-9, 1995). The data presented above indicate immunization strategies involving the combination of GM-CSF and **CpG** oligonucleotide are particularly effective. **CpG** oligonucleotide and soluble GM-CSF were only additive in their ability to induce anti-IdIgG after immunization with Id-KLH which may have. . .

DETD **CpG** Oligonucleotide Enhances Production of Anti-Id Antibodies Following Immunization with Id/GM-CSF Fusion Protein

DETD . . . shown to be an excellent immunogen (Tao, M. H., and Levy, R., Nature 362:755-758, 1993). In order to evaluate if **CpG** oligonucleotide can further enhance the specific antibody response induced by Id/GM-CSF, mice were immunized with Id-KLH or Id/GM-CSF with and without **CpG** oligonucleotide as an adjuvant. Serum was obtained weekly and anti-Id IgG levels determined. No toxicity was observed in any mice. As illustrated in FIG. 2, **CpG** oligonucleotide enhanced production of anti-Id antibodies in response to Id/GM-CSF.

DETD . . . immunized on day 0 and boosted on day 14 with the same antigen and adjuvant. The combination of Id/GM-CSF and **CpG** oligonucleotide induced remarkably high levels of anti-Id IgG after two immunizations (FIG. 3). Serum obtained 1 week after the final . . . GM-CSF sequences were replaced with human GM-CSF sequences. Levels of anti-Id produced after immunization using Id/human GM-CSF with or without **CpG** oligonucleotide were significantly lower than those seen following Id/GM-CSF and similar to those seen with Id-KLH, demonstrating that biologically active. . .

DETD **CpG** Oligonucleotide Enhances Production of Antigen Specific Antibody of IgG_{2a} Isotype

DETD . . . IgG_1 and IgG_{2a} was assessed following immunization (FIG. 4). Immunization included various combinations of Id-KLH or Id/GM-CSF with GM-CSF or **CpG** oligonucleotide. Serum was sampled 4 weeks after a single immunization. **CpG** oligonucleotide induced enhanced production of anti-Id IgG_{2a} compared with that seen under the corresponding conditions without **CpG** oligonucleotide. Similar IgG_1 /IgG_{2a} ratios were seen at other time points.

DETD Immunization Using **CpG** Oligonucleotide and ID/GM-CSF Fusion Protein Further Protection of Mice From Tumor Growth

DETD In order to evaluate whether **CpG** oligonucleotide can also serve as an effective adjuvant with Id/GM-CSF immunization, mice were challenged with tumor three days after a single immunization with Id/GM-CSF with or without **CpG** oligonucleotide. Immunization using this schedule was only minimally effective with Id-KLH. **CpG** oligonucleotide 1758 and **CpG** oligonucleotide 1826 were equally effective at prolonging survival when used alone or in combination with Id/GM-CSF. The data illustrated in FIG. 5 represents the combined results of mice treated with **CpG** oligonucleotide 1758 and **CpG** oligonucleotide 1826. All unimmunized mice, and mice treated with **CpG** oligonucleotide without antigen, developed tumor and died within 50 days. Thirty percent of mice immunized with I/GM-CSF alone remained disease free, whereas 70% of the group immunized with Id/GM-CSF and **CpG** oligonucleotide remained disease free. Mice immunized with Id/GM-CSF and **CpG** oligonucleotide had survival that was statistically superior to that seen with no immunization or treatment with **CpG** oligonucleotide alone ($P<0.001$).

The difference between those immunized with Id/GM-CSF alone versus those immunized with **CpG** oligonucleotide plus Id/GM-CSF approached statistical significance (P-0.072).

DETD . . . and in the studies of Example 5, remarkable levels of anti-Id IgG were achieved after repeated immunization with Id/GM-CSF and **CpG** oligonucleotide. **CpG** oligonucleotide shifted the response to a IgG_{sa} under all conditions studied including immunization with soluble GM-CSF and the Id/GM-CSF fusion. . . Th1 response. Immunization using this approach translated into protection from tumor growth only 3 days after immunization with Id/GM-CSF and **CpG** oligonucleotide. This is the most effective protection reported to date in this extensively studied model.

DETD **CpG** Oligonucleotide Effects on Dendritic Cell Phenotype

DETD The synergistic effects of **CpG** oligonucleotide and GM-CSF suggested the possibility that these agents together may enhance expression of costimulatory molecules or MHC by APCs.. . . of these molecules by bone-marrow derived dendritic cells was evaluated. Flow cytometric analysis of dendritic cells pulsed with Id/GM-CSF and/or **CpG** oligonucleotide demonstrated a modest increase in expression of class I and class II MHC in response to the combination of Id/GM-CSF and **CpG** oligonucleotide. Baseline expression of CD80 and CD86 expression was high, and was not altered extensively by Id/GM-CSF or **CpG** oligonucleotide (FIG. 6).

DETD **CpG** Oligonucleotide Enhances Production of IL-12 By Dendritic Cells Pulsed With Id/GM-CSF

DETD The enhanced Th1 response to antigen could be explained by the ability of **CpG** oligonucleotide to enhance production of IL-12 by APCs such as dendritic cells. The production of IL-12 by bone-marrow derived dendritic cells that were pulsed with antigen, including Id/GM-CSF, was assessed in the presence of **CpG** oligonucleotide. As illustrated in FIG. 7, pulsing of dendritic cells with **CpG** oligonucleotide increased production of IL-12, particularly when cells were also pulsed with Id/GM-CSF. IL-6 production by dendritic cells was also increased by the addition **CpG** oligonucleotide to Id/GM-CS, although the effect was less pronounced than for IL-12. The impact of GM-CSF alone on dendritic cell. . . not studied since these cells were generated using GM-CSF. The markedly enhanced production of IL-12 by dendritic cells induced by **CpG** oligonucleotide may at least in part explain the enhanced Th1 response.

DETD The ability of a **CpG** oligonucleotide to induce IL-12 secretion is a good measure of its adjuvant potential, especially in terms of its ability to. . . IL-12 secretion from human PBMC in vitro (Table 1) was examined. These experiments showed that in some human PBMC, most **CpG** oligonucleotide could induce IL-12 secretion (e.g., expt. 1). However, other donors responded to just a few **CpG** oligonucleotide (e.g., expt. 2). Oligonucleotide 2006 was a consistent inducer of IL12 secretion from most subjects (Table 2).

DETD TABLE 2

Induction of human IL-12 secretion by Phosphorothioate **CpG** oligonucleotide

		IL-12 (pg/ml)	
ODN_1	sequence (5'-3')	expt. 1	expt. 2
None		0	0
1962	TCCTGTCGTTCCTTGTCGTT (SEQ. ID NO:79)	19	0
1965	TCCTGTCGTTTTTTGTCGTT (SEQ. ID NO:81). . .		

DETD **CpG** and GM-CSF Synergistically Increase Co-Stimulatory Molecules on DC

DETD . . . immune response by DC. Functional activation of DC requires by the expression of co-stimulatory molecules. We examined the effect of **CpG** on the expression of the intercellular adhesion molecule-1 (ICAM-1, CD54), and the co-stimulatory surface molecules B7-2 (CD86) and CD40. First,. . . 9, panel C) was quantified in flow cytometry by the mean fluorescence intensity (MFI) of viable DC. In all experiments, **CpG** was superior to GMCSF in enhancing expression of co-stimulatory molecules. Compared to the cells only sample, the **CpG** oligonucleotide 2006 enhanced the expression of CD54 (25.0+-5.7 vs. 7.0+-1.8; p=0.02, n=5), CD 86 (3.9+-0.8 vs. 1.6+-0.3; p=0.01; n=5) and. . .

DETD Specificity was tested using 2117 (methylated version of 2006) and 2078 (GpC version of 2080). The non-**CpG** oligonucleotide 2117 showed no synergistic enhancement of CD40 expression when combined with GMCSF. An assay was performed on primary dendritic. . .

DETD TABLE 3

Compound	CD86 (5 Exp)	CD40 (4 Exp.)	T cell proliferation
GM-CSF	1.9	2.5	13.3
CpG	3.9	3.5	19.7
CpG + GM-CSF	7.0	8.5	25.6

DETD . . . TGTCGTTGTCGTTGTCGTT (SEQ ID NO: 96)

TCGTCGTCGTCGTT (SEQ ID NO: 97)

TGTCGTTGTCGTT (SEQ ID NO: 98)

TCCATAGCGTTCCTAGCGTT (SEQ ID NO: 99)
TCCATGACGTTCCTGACGTT (SEQ ID NO: 100)
GTCG(T/C)T (SEQ ID NO: 101)
TGTCG(T/C)T (SEQ ID NO: 102)
TCCATGAGCTTCCTGAGTCT (SEQ ID NO: 103)
TCTCCCAGCGTGCGCCAT (SEQ ID NO: 104)
TCCATGACGTTCCTGACGTT (SEQ ID NO: 105)

. . . specific immune response of an immunopotentiating cytokine selected from the group consisting of GM-CSF, IL-2, IL-4 and IFN-γ, and an **immunostimulatory CpG** oligonucleotide having a sequence including at least the following formula: 5' X_1 CGX_2 3' wherein the oligonucleotide includes at least. . . nucleotides, wherein the cytokine is a peptide, whereby an antigen is optionally additionally administered, and wherein the antigen and the **CpG** oligonucleotide are not conjugated.

6. The method of claim 1, wherein the antigen is administered to the subject in conjunction with the **immunostimulatory CpG** oligonucleotide and the immunopotentiating cytokine.

12. A composition, comprising: an effective amount for synergistically activating a dendritic cell of an **immunostimulatory CpG** oligonucleotide having a sequence including at least the following formula: 5' X_1 CGX_2 3' wherein the oligonucleotide includes at least. . .

14. The composition of claim 12, further comprising an antigen and wherein the antigen and the **CpG** oligonucleotide are not conjugated.

. . . a dendritic cell of an immunopotentiating cytokine selected from the group consisting of GM-CSF, IL-2, IL-4 and IFN-γ, and an **immunostimulatory CpG** oligonucleotide having a sequence including at least the following formula: 5' X_1 CGX_2 3' wherein the oligonucleotide includes at least. . . nucleotides, wherein the cytokine is a peptide, whereby an antigen is optionally additionally administered, and wherein the antigen and the **CpG** oligonucleotide are not conjugated.

. . . having a neoplastic disorder an immunopotentiating cytokine selected from the group consisting of GM-CSF, IL-2, IL-4 and IFN-γ, and an **immunostimulatory CpG** oligonucleotide having a sequence including at least the following formula: 5' X_1 CGX_2 3' wherein the oligonucleotide includes at least. . . nucleotides, in an amount effective for synergistically increasing survival time of the subject with respect to a subject administered the **immunostimulatory CpG** oligonucleotide or the immunopotentiating cytokine alone, where the cytokine is a peptide.

20. The method of claim 18, wherein the **immunostimulatory CpG** oligonucleotide and the immunopotentiating cytokine are injected directly into the tumor.

L15 ANSWER 9 OF 12 USPATFULL on STN
2001:52030 Use of nucleic acids containing unmethylated CPC dinucleotide in the treatment of LPS-associated disorders.
Krieg, Arthur M., Iowa City, IA, United States
Schwartz, David A., Iowa City, IA, United States
University of Iowa Research Foundation, Iowa City, IA, United States (U.S. corporation)
US 6214806 B1 20010410
APPLICATION: US 1998-30701 19980225 (9)
PRIORITY: US 1997-39405P 19970228 (60)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. A method of treating a subject having or at risk of having an acute decrement in air flow, comprising: administering to a subject having or at risk of having an acute decrement in air flow, wherein the acute decrement in air flow results from endotoxin exposure, a therapeutically effective amount of a nucleic acid sequence containing at least one unmethylated **CpG**.

2. The method of claim 1, wherein the nucleic acid sequence is from 8-30 bases in length.

3. The method of claim 1, wherein the subject is human.

4. The method of claim 1, wherein the acute decrement in airflow results from lipopolysaccharide (LPS) exposure.

5. The method of claim 1, wherein the nucleic acid sequence has a formula: 5'N_1 X_1 CGX_2 N_2 3' (SEQ ID NO:1) wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymidine; X_2 is cytosine or thymine, N is any nucleotide and N_1 +N_2 is from about 0-26 bases.

6. The method of claim 5, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.

7. The method of claim 5, wherein said nucleic acid sequence is SEQ ID NO:2.

8. The method of claim 1, wherein the nucleic acid sequence has a formula: 5'N_1 X_1 X_2 CGX_3 X_4 N_2 3' (SEQ ID NO:3) wherein at least one nucleotide separates consecutive CpGs; X_1 X_2 is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X_3 X_4 is selected from the group consisting of TpT or CpT; N is any nucleotide and N_1 +N_2 is from about 0-26 bases.

9. The method of claim 8, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.

10. The method of claim 1 wherein the endotoxin exposure results from inhalation of LPS.

11. The method of claim 10 wherein the endotoxin exposure results in dust-induced airway disease.

12. The method of claim 10 wherein the endotoxin exposure results in LPS-induced asthma.

13. The method of claim 1 wherein the endotoxin exposure results in adult respiratory distress syndrome (ARDS).

14. The method of claim 1 wherein the endotoxin exposure results in endotoxemia.

15. The method of claim 1 wherein the endotoxin exposure results in systemic inflammatory response syndrome SIRS.

16. The method of claim 1 wherein the endotoxin exposure results in sepsis syndrome.

17. The method of claim 1 wherein the endotoxin exposure results in septic shock.

18. The method of claim 1 wherein the endotoxin exposure results in disseminated intravascular coagulation (DIC).

19. The method of claim 1 wherein the endotoxin exposure results in cardiac dysfunction.

20. The method of claim 1 wherein the endotoxin exposure results in organ failure, wherein the organ failure is selected from the group consisting of liver failure, brain failure, renal failure, and multi-organ failure.

21. The method of claim 1 wherein the endotoxin exposure results from a route of administration selected from the group consisting of administration of LPS-contaminated fluids and gram-negative infections.

22. The method of claim 1 wherein the subject is a subject who has been treated with chemotherapy.

23. The method of claim 1 wherein the subject is an immunocomprised subject.

24. The method of claim 1 wherein the nucleic acid sequence containing at least one unmethylated **CpG** is administered by a route selected from the group consisting of intravenous, parenteral, oral, implant and topical.

25. A method of inhibiting an inflammatory response in a subject having inhaled or at risk of having inhaled lipopolysaccharide (LPS),

comprising: administering to a subject having inhaled or at risk of having inhaled LPS, a therapeutically effective amount for inhibiting an inflammatory response of a nucleic acid sequence containing at least one unmethylated **CpG**.

26. The method of claim 25, wherein the nucleic acid sequence is from 8-30 bases in length.

27. The method of claim 25, wherein the subject is human.

28. The method of claim 25, wherein the nucleic acid sequence has a formula: 5'N_1 X_1 CGX_2 N_2 3' (SEQ ID NO:1) wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymidine; X_2 is cytosine or thymine, N is any nucleotide and N_1 +N_2 is from about 0-26 bases.

29. The method of claim 28, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.

30. The method of claim 28, wherein said nucleic acid sequence is SEQ ID NO:2.

31. The method of claim 25, wherein the nucleic acid sequence has a formula: 5'N_1 X_1 X_2 CGX_3 X_4 N_2 3' (SEQ ID NO:3) wherein at least one nucleotide separates consecutive CpGs; X_1 X_2 is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X_3 X_4 is selected from the group consisting of TpT or CpT; N is any nucleotide and N_1 +N_2 is from about 0-26 bases.

32. The method of claim 31, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.

33. A method of modifying the level of a cytokine in a subject having inhaled or at risk of having inhaled lipopolysaccharide (LPS), comprising: administering to a subject having inhaled or at risk of having inhaled LPS a therapeutically effective amount for modifying the level of a cytokine of a nucleic acid sequence containing at least one unmethylated **CpG** dinucleotide.

34. The method of claim 33, wherein the nucleic acid sequence is from 8-30 bases in length.

35. The method of claim 33, wherein the subject is human.

36. The method of claim 33, wherein said nucleic acid sequence is SEQ ID NO:2.

37. The method of claim 33, wherein the nucleic acid sequence has a formula: 5'N_1 X_1 CGX_2 N_2 3' (SEQ ID NO:1) wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymidine; X_2 is cytosine or thymine, N is any nucleotide and N_1 +N_2 is from about 0-26 bases.

38. The method of claim 37, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.

39. The method of claim 33, wherein the nucleic acid sequence has a formula: 5'N_1 X_1 X_2 CGX_3 X_4 N_2 3' (SEQ ID NO:3) wherein at least one nucleotide separates consecutive CpGs; X_1 X_2 is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X_3 X_4 is selected from the group consisting of TpT or CpT; N is any nucleotide and N_1 +N_2 is from about 0-26 bases.

40. The method of claim 39, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.

41. The method of claim 33, wherein said modulation is a reduction in the level of said cytokine.

42. The method of claim 33, wherein said modulation is an increase in the level of said cytokine.

43. The method of claim 33, wherein said cytokine is selected from the group consisting of TNF-α, MIP-2, IL-10, IL-12, and interferon-γ.

AI **US 1998-30701 19980225 (9)**

AB The present invention is based on the finding that nucleic acids containing at least one unmethylated cytosine-guanine (**CpG**) dinucleotide affect immune responses in a subject. These nucleic acids containing at least one unmethylated cytosine-guanine (**CpG**) dinucleotide can be used to treat pulmonary disorders having an immunologic component, such as a response to inhaled lipopolysaccharide. The. . .

SUMM This invention relates to generally to pulmonary disorders, and specifically to the use of oligonucleotides having at least one unmethylated **CpG** dinucleotide (**CpG** ODN) in the treatment of such disorders.

SUMM The present invention is based on the finding that nucleic acids containing at least one unmethylated cytosine-guanine (**CpG**) dinucleotide affect the immune response in a subject by activating natural killer cells (NK) or redirecting a subject's immune response. . . Th1 response by inducing monocytic and other cells to produce Th1 cytokines. These nucleic acids containing at least one unmethylated **CpG** can be used to treat pulmonary disorders having an immunologic component, such as asthma or environmentally induced airway disease.

SUMM . . . an acute decrement in air flow by administering a therapeutically effective amount of nucleic acids containing at least one unmethylated **CpG** is provided.

SUMM . . . having an inflammatory response to lipopolysaccharide by administering a therapeutically effective amount of nucleic acids containing at least one unmethylated **CpG** is also provided. The invention also provides a method of modifying the level of a cytokine in a subject having or at risk of having inhaled lipopolysaccharide by administering a therapeutically effective nucleic acid containing at least one unmethylated **CpG**.

SUMM . . . at risk of having an inflammatory response to inhaled lipopolysaccharide including a nucleic acid sequence containing at least one unmethylated **CpG** in a pharmacologically acceptable carrier.

DRWD . . . (TNF-α, MIP-2, IL-10, IL-12, and IFN-γ) in the serum four hours after intravenous treatment with either an oligonucleotide containing embedded **CpG** motifs or an oligonucleotide without **CpG** motifs. Serum samples were obtained immediately following an inhalation challenge with E. coli LPS. Error bars show Standard Error (SE).

DRWD . . . minutes, four hours and 12 hours prior to the inhalation challenge, mice were either treated with an oligonucleotide containing embedded **CpG** motifs or were treated with an oligonucleotide without **CpG** motifs. Error bars show SE.

DRWD . . . in the whole lung lavage fluid following inhalation of E. coli LPS. Mice were pretreated with an oligonucleotide containing embedded **CpG** motifs or were pretreated with an oligonucleotide without **CpG** motifs four hours prior to inhalation challenge with LPS. Error bars show SE.

DRWD . . . isolated from lungs of mice exposed to E. coli LPS by inhalation. Mice were pretreated with an oligonucleotide containing embedded **CpG** motifs or were pretreated with an oligonucleotide without **CpG** motifs four hours prior to inhalation challenge with LPS. L32 encodes a ribosomal protein and was used to assess the. . .

DRWD . . . in the whole lung lavage fluid following inhalation of E. coli LPS. Mice were pretreated with an oligonucleotide containing embedded **CpG** motifs or were pretreated with an oligonucleotide without **CpG** motifs four hours prior to inhalation challenge with LPS. Error bars show SE.

DRWD . . . inhalation of E. coli LPS. C57BL/6 mice and IL-10 knockout mice (C57BL/6-IL10^{tm1Cgn}) were pretreated with either an oligonucleotide containing embedded **CpG** motifs or with intravenous saline four hours prior to inhalation challenge with LPS. Error bars show SE.

DRWD . . . egg antigen "SEA" (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given **CpG** ODN along with egg, the inflammatory cells in the lung are not as increased by subsequent inhalation of SEA (open. . .

DRWD . . . and subsequently inhale SEA (open circle), many eosinophils are present in the lungs. However, when the mice are initially given **CpG** ODN along with egg, the inflammatory cells in the lung are not as increased by subsequent inhalation of the SEA. . .

DRWD . . . the percentage of macrophage, lymphocyte, neutrophil and eosinophil cells induced by exposure to saline alone; egg, then SEA; egg and **CpG** ODN, then SEA; and egg and control oligo, then SEA. When the mice are treated with the control oligo at. . . inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs.

However, giving a **CpG** oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes. . .

DRWD . . . 4 (IL-4) production pg/ml) in mice over time in response to injection of egg, then SEA (open diamond); egg and **CpG** ODN, then SEA (open circle); or saline, then saline (open square). The graph shows that the resultant inflammatory response correlates. . .

DRWD . . . plotting interleukin 12 (IL-12) production (pg/ml) in mice over time in response to injection of saline; egg, then SEA; or **CpG** ODN and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can actually redirect the cytokine response of the lung to production of IL-12, indicating a Th1 type of immune. . .

DRWD . . . plotting interferon gamma (IFN-γ production (pg/ml) in mice over time in response to injection of saline; egg, then saline; or **CpG** ODN and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune. . .

DETD . . . 1:161, 1991). The present invention is based on the finding that certain oligonucleotides (ODN) containing at least one unmethylated cytosine-guanine (**CpG**) dinucleotide activate the immune response.

DETD . . . decrement in air flow by administering a therapeutically effective amount of a nucleic acid sequence containing at least one unmethylated **CpG**. The term "nucleic acid" or "oligonucleotide" refers to a polymeric form of nucleotides at least five bases in length. The. . .

DETD . . . end of the nucleic acid. International Patent Application WO 95/26204, entitled "Inmune stimulation by phosphorothioate oligonucleotide analogs" reports the nonsequence-specific **immunostimulatory** effect of phosphorothioate modified oligonucleotides. Nontraditional bases such as inosine and queosine, as well as acetyl-, thio- and similarly modified. . .

DETD A "**CpG**" or "**CpG** motif" refers to a nucleic acid having a cytosine followed by a guanine linked by a phosphate bond. The term "methylated **CpG**" refers to the methylation of the cytosine on the pyrimidine ring, usually occurring the 5-position of the pyrimidine ring. The term "unmethylated **CpG**" refers to the absence of methylation of the cytosine on the pyrimidine ring. Methylation, partial removal, or removal of an unmethylated **CpG** motif in an oligonucleotide of the invention is believed to reduce its effect. Methylation or removal of all unmethylated **CpG** motifs in an oligonucleotide substantially reduces its effect. The effect of methylation or removal of a **CpG** motif is "substantial" if the effect is similar to that of an oligonucleotide that does not contain a **CpG** motif.

DETD Preferably the **CpG** oligonucleotide is in the range of about 8 to 30 bases in size. For use in the instant invention, the. . . Let. 29:2619-2622, 1988). These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, **CpG** dinucleotides can be produced on a large scale in plasmids, (see Sambrook, T., et al., Molecular Cloning: A Laboratory Manual,. . .

DETD . . . or more than one CCG or CGG trimer at or near the 5' or 3' terminals and/or the consensus mitogenic **CpG** motif is not a palindrome. A "palindromic sequence" or "palindrome" means an inverted repeat (i.e., a sequence such as ABCDEE'D'C'B'A',. . .

DETD In another embodiment, the method of the invention includes the use of an oligonucleotide which contains a **CpG** motif represented by the formula:

DETD . . . a preferred embodiment, N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer. **CpG** ODN are also preferably in the range of 8 to 30 bases in length, but may be of any size. . . or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus mitogenic **CpG** motif is not a palindrome. Other **CpG** oligonucleotides can be assayed for efficacy using methods described herein.

DETD . . . example, at the last five nucleotides of the 3' end of the nucleic acid. Preferred nucleic acids containing an unmethylated **CpG** have a relatively high stimulation with regard to B cell, monocyte, and/or natural killer cell responses (e.g., induction of cytokines,. . .

DETD The "stimulation index" is a measure of a **CpG** ODN to effect an immune response which can be tested in various immune cell assays. The stimulation of the immune. . . or at risk of having an acute decrement in air flow in response to endotoxin, it is important that the **CpG** ODN be capable of effectively inducing cytokine secretion by monocytic cells and/or Natural Killer (NK) cell lytic activity. In one method, the stimulation index of the **CpG** ODN with regard to B-cell proliferation is at least about 5, preferably at least about 10, more preferably at least. . .

DETD The **CpG** ODN of the invention stimulate cytokine production (e.g., IL-6, IL-12, IFN-γ, TNF-α and GM-CSF). Exemplary sequences include:

DETD The **CpG** ODN of the invention are also useful for stimulating natural killer cell (NK) lytic activity in a subject such as. . .

DETD Preferred **CpG** ODN can effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml of. . . indication. These cytokines can be measured by assays well known in the art. The ODNs listed above or other preferred **CpG** ODN can effect at least about 10%, more preferably at least about 15% and most preferably at least about 20%. . .

DETD An oligonucleotide containing at least one unmethylated **CpG** can be used alone to activate the immune response or can be administered in combination with another therapeutic modality, either a drug or a surgical procedure. For example, when the oligonucleotide containing at least one unmethylated **CpG** is administered in conjunction with another therapeutic modality, the oligonucleotide can be administered before, after, and/or simultaneously with the other therapeutic modality. The oligonucleotide containing at least one unmethylated **CpG** can have an additional efficacy (e.g., through antisense or other means) in addition to its ability to activate the immune. . .

DETD . . . LPS by administering to the subject a therapeutically effective amount of a nucleic acid sequence containing at least one unmethylated **CpG**.

DETD . . . J. Exp. Med. 178:1041-1048, 1993). Without wanting to be bound by theory, it is possible that nucleic acids containing unmethylated **CpG** could reduce the inflammatory response to LPS by increasing the production and response of IL-10, or by modulating the response. . .

DETD . . . cytokine when it is underexpressed. Modulation of a particular cytokine can occur locally or systemically. It is believed that the **CpG** oligonucleotides do not directly activate purified NK cells, but rather render them competent to respond to IL-12 with a marked increase in their IFN-γ production. By inducing IL-12 production and the subsequent increased IFN-γ secretion by NK cells, the **immunostimulatory** nucleic acids also promote a Th1 type immune response. No direct activation of proliferation or cytokine secretion by highly purified. . .

DETD In the first series of experiments, mice were intravenously treated with 20 base pair (bp) oligonucleotides containing **CpG** motifs (**CpG** oligo) or 20 bp oligonucleotides without embedded **CpG** motifs (non **CpG** oligo) 30 min, 4 hours, or 12 hours prior to a 4 hour inhalation challenge with E. coli LPS (1.5 μg/m^3). To determine whether unmethylated **CpG** motifs were responsible for the protective effect, we pretreated mice with oligonucleotides containing either unmethylated **CpG** motifs or methylated **CpG** motifs prior to an inhalation challenge with E. coli LPS. Finally, to determine the role of IL-10, we pretreated IL-10 knockout mice with **CpG** oligos and then performed a similar inhalation challenge with E. coli LPS. Immediately post inhalation challenge, all mice were sacrificed,. . .

DETD Oligonucleotides. Twenty base pair oligonucleotides were synthesized with and without the embedded **CpG** motifs (Oligos etc., Wilsonville, Oreg.). These oligonucleotides contained a nuclease-resistant phosphorothioate-modified backbone, and were purified by two rounds of ethanol precipitation prior to use. The **CpG** dinucleotide was flanked by two 5' purines and two 3' pyrimidines to enhance the stimulatory effect of the oligonucleotide.

DETD The "nonstimulatory" oligonucleotide was identical to the stimulatory oligonucleotide except that the two embedded **CpG** motifs were modified, one appearing as an ApG motif and the other appearing as a GpC motif. The two synthesized. . .

DETD **CpG** Oligonucleotide: ATAATCGACGTTCAAGCAAG (SEQ ID NO:2)

DETD Non-**CpG** oligonucleotide: ATAATAGAGCTTCAAGCAAG (SEQ ID NO:18)

DETD . . . Protocol. DNA was methylated as we have described previously (Krieg, A. M., et al., Nature 374:546-9, 1995) with 2 U **CpG** methylase (New England Biolabs; Beverly, Mass.) per μg DNA for 18 hours at 37° C. Methylated DNA was tested to. . .

DETD Statistical Analysis. Three comparisons were pursued in this analysis: 1) the effect of intravenous **CpG** containing oligonucleotides versus oligonucleotides without embedded **CpG** motifs in modulating the inflammatory response to inhaled LPS; 2) the effect of unmethylated **CpG** motifs versus methylated **CpG** motifs in controlling the inflammatory response to LPS; and 3) the role of IL-10 in mediating the protective effect of unmethylated **CpG** containing oligonucleotides. The inflammatory response was assessed using lavage cellularity, lavage fluid cytokine concentration, serum concentration of cytokines, and the. . .

DETD **CpG** ODN Reduces the Pulmonary Response to Inhaled LPS and Stimulates the Immune Response

DETD Pretreatment with **CpG** oligonucleotides (ODN) resulted in a systemic

inflammatory response. Although intravenous treatment with **CpG** ODN did not affect the concentration of peripheral white blood cells; compared to non-**CpG** ODN, treatment with **CpG** ODN prior to LPS inhalation resulted in a higher concentration of PMNs 30 min, 4 hours, and 12 hours after injection. As expected, intravenous treatment with **CpG** oligonucleotides also affected the concentration of cytokines in the serum.

DETD Compared to non-**CpG** ODN, **CpG** ODN resulted in an increase in the concentration of MIP-2, IL-10, and IL-12 in the serum of mice following LPS. . . . were most pronounced 30 min and 4 hours after intravenous administration but were still present 12 hours after exposure to **CpG** containing oligonucleotides. No differences were observed for the serum concentration of TNF-α, IL-6, and IFN-γ at any of the time.

. .

DETD Pretreatment with **CpG** containing oligonucleotides reduced the pulmonary response to inhaled LPS. Animals pretreated with **CpG** oligonucleotides at 0.5, 4, and 12 hours had a reduced concentration of cells in the lavage fluid following inhalation challenge. . . . 12 hours prior to the inhalation challenge did not affect the percentage of lavage PMNs (FIG. 3). Although pretreatment with **CpG** containing oligonucleotides resulted in significant changes in the concentration of cytokines in the lavage fluid, the changes in cytokine concentration were predominantly evident when mice were pretreated with **CpG** oligonucleotides 4 hours prior to the inhalation challenge. While significant reductions were observed in the concentration of TNF-α and MIP-2, the lavage fluid concentration of IL-12 was elevated following treatment with **CpG** oligonucleotides 4 hours prior to the inhalation challenge (FIG. 4). IL-6, IL-10, and IFN-γ were not measurable in the lavage. . . indicate that total lung mRNA concentrations for TNF-α, MIP-2, IL-6, IL-10, and IFN-γ are similar in mice pretreated with the **CpG** and non-**CpG** containing oligonucleotide (FIG. 5). These results also demonstrate that mRNA IL-12 appears to be upregulated in the lung only from mice pretreated with **CpG** containing oligonucleotides.

DETD To determine the specificity of the **CpG** oligonucleotides in suppressing the inflammatory response to inhaled LPS, the **CpG** motifs were methylated. The immunosuppressive effects of two identical oligonucleotides, one with unmethylated **CpG** motifs and the other with methylated **CpG** motifs, were compared. Methylating the **CpG** motifs abolished the protective effect of **CpG** oligonucleotides in preventing the cellular inflammatory response to inhaled LPS (FIG. 6).

DETD . . . ATAATCGACGTTCAAGCAAG (SEQ ID NO:2)

1631 CGCGCGCGCGCGCGCGCGCG (SEQ ID NO:59)

1835 TCTCCCAGCGAGCGCCAT (SEQ ID NO:60)

1759 ATAATCCAGCTTGAACCAAG (SEQ ID NO:61)

1826 **TCCATGACGTTCCTGACGTT** (SEQ ID NO:62)

1585 GGGGTCAACGTTGAGGGGGG (SEQ ID NO:63)

2010 GCGGCGGGCGGCGCGCGCCC (SEQ ID NO:54)

1972 GGGGTCTGTGCTTTTGGGGGG (SEQ ID NO:64)

2001 GGCGGCGGCGGCGGCGGCGG. . .

DETD . . . fit the motifs shown in SEQ ID NO:1 and SEQ ID NO:3. Oligonucleotides such as 2001 and 2010, which contain **CpG** motifs with CCGG, CCG, and CGG, can also have a beneficial effect.

DETD The results indicate that **CpG** containing oligonucleotides substantially reduce the inflammatory response to inhaled LPS and that the protective effect appears to be specific to unmethylated **CpG** motifs embedded within the oligonucleotide. These findings suggest that oligonucleotides containing **CpG** motifs may prove helpful in controlling the inflammatory response to inhaled LPS and other environmental agents.

DETD . . . J., et al., J. Clin. Invest. 96:2339-2347, 1995), IL-10 might play a critical role in mediating the immunosuppressive effects of **CpG** oligonucleotides. To pursue this hypothesis, IL-10 knockout (C57BL/6-Il10^{tm1Cgn}) mice and C57BL/6 control mice were pretreated with **CpG** containing oligonucleotides and then an inhalation challenge with E. coli LPS was performed. Compared to pretreatment with intravenous saline, **CpG** containing oligonucleotides significantly reduced the total cellularity and the concentration of PMNs in the lavage fluid in both C57BL/6 and mice with a disrupted IL1-10 gene (C57BL/6-Il10^{tm1Cgn}) (FIG. 7). Importantly, the immunosuppressive effects of **CpG** oligonucleotides were equally effective in mice with a disrupted IL-10 gene compared to wild type mice.

DETD The results indicate that the protective effect of unmethylated **CpG** motifs is not dependent on IL-10.

DETD . . . at 5×10^6 /well, at 37° C. in a 5% CO_2 humidified atmosphere in 24-well plates with medium alone or with **CpG** or non-**CpG** ODN at the indicated concentrations, or with E. coli or calf thymus (50 μg/ml) at 37° C. for 24 hr.. . .

DETD Experiments were conducted to determine whether **CpG** containing

oligonucleotides stimulated the activity of natural killer (NK) cells in addition to B cells. As shown in Table 2,. . .

DETD **CpG** ODN 1: GCTAGACGTTAGCGT (SEQ ID NO:19)

DETD (where X=5 methyl cytosine) was observed. In contrast, there was relatively no induction in effectors that had been treated with non-**CpG** control ODN.

DETD TABLE 2

Induction Of NK Activity By **CpG** Oligodeoxynucleotides (ODN)

	% YAC-1 Specific Lysis* Effector: Target			% 2C11 Specific Lysis Effector: Target	
ODN	50:1		100:1	50:1	100:1
None	-1.1	-1.4	15.3	16.6	
1	16.1		24.5	38.7	47.2
3Dd	17.1		27.0	37.0	40.0
non-**CpG** ODN	-1.6	-1.7	14.8	15.4	

DETD Induction of NK activity by DNA containing **CpG** motifs, but not by non-**CpG** DNA.

DETD . . . 3). To determine whether the stimulatory activity of bacterial DNA may be a consequence of its increased level of unmethylated **CpG** dinucleotides, the activating properties of more than 50 synthetic ODN containing unmethylated, methylated, or no **CpG** dinucleotides was tested. The results, summarized in Table 3, demonstrate that synthetic ODN can stimulate significant NK activity, as long as they contain at least one unmethylated **CpG** dinucleotide (Ballas, Z., et al., J. Immunol 157:1840-1845, 1996). No difference was observed in the stimulatory effects of ODN in which the **CpG** was within a palindrome (such as ODN 1585, which contains the palindrome AACGTT) from those ODN without palindromes (such as 1613 or 1619), with the caveat that optimal stimulation was generally seen with ODN in which the **CpG** was flanked by two 5' purines or a 5' GpT dinucleotide and two 3' pyrimidines. Kinetic experiments demonstrated that NK. . . of the ODN. The data indicates that the murine NK response is dependent on the prior activation of monocytes by **CpG** DNA, leading to the production of IL-12, TNF-α, and IFN.

DETD TABLE 3

Induction of NK Activity by DNA Containing **CpG** Motifs but not by Non-**CpG** DNA

	DNA or Cytokine Added		LU/10^6
		Human Cells	Mouse Cells
Expt. 1	None	0.00	0.00
	IL-2	16.68	15.82
	E. Coli DNA. . . NO:23)	5.22	
	1769 -------X----- (SEQ ID NO:24)	0.02	ND
	1613 TCCATG.CGTTCCTGATGCT (SEQ ID NO:5)	3.35	
	1765 --------X---------- (SEQ ID NO:25)	0.11	

CpG dinucleotides in ODN sequences are indicated by underlining; X indicates methylcytosine. Lower case letters indicate nuclease resistant phosphorothioate modified internucleotide. . .

DETD Immune activation by **CpG** motifs may depend on bases flanking the **CpG**, and the number and spacing of the CpGs present within an ODN. Although a single **CpG** in an ideal base context can be a very strong and useful immune activator, superior effects can be seen with ODN containing several CpGs with the appropriate spacing and flanking bases. For activation of murine B cells, the optimal **CpG** motif is TGACGTT.

DETD . . . ODN sequences for stimulation of human cells by examining the effects of changing the number, spacing, and flanking bases of **CpG** dinucleotides.

DETD Identification of phosphorothioate ODN with optimal **CpG** motifs for activation of human NK cells

DETD . . . cellular uptake (Krieg et al., Antisense Res. Dev. 6:133, 1996) and improved B cell stimulation if they also have a **CpG** motif. Since NK activation correlates strongly with in vivo adjuvant effects, the identification of phosphorothioate ODN that will activate human. . .

DETD The effects of different phosphorothioate ODNs, which contain **CpG** dinucleotides in various base contexts, on human NK activation (Table 4) were examined. ODN 1840, which contained 2 copies of. . . 4). To further identify additional ODNs optimal for NK activation, approximately one hundred ODN containing different numbers and spacing of **CpG** motifs, were tested with ODN 1982 serving as a control. Sample results are shown in Table 5.

DETD . . . generally began with a TC or TG at the 5' end, however, this requirement was not mandatory. ODNs with internal **CpG** motifs (e.g., ODN 1840) are generally less potent stimulators than those in which a GTCGCT (SEQ ID NO:49) motif immediately. . . in which only one of the motifs had the addition of the spacing two Ts. The minimal acceptable

spacing between **CpG** motifs is one nucleotide as long as the ODN has two pyrimidines preferably T) at the 3' end (e.g., ODN. . . T also created a reasonably strong inducer of NK activity (e.g., ODN 2016). The choice of thymine (T) separating consecutive **CpG** dinucleotides is not absolute, since ODN 2002 induced appreciable NK activation despite the fact that adenine (a) separated its CpGs (i.e., CGACGTT (SEQ ID NO:57)). It should also be noted that ODNs containing no **CpG** (e.g., ODN 1982), runs of CpGs, or CpGs in bad sequence contexts (e.g., ODN 2010) had little or no stimulatory. . .

DETD TABLE 5

Induction of NK LU by Phosphorothioate **CpG** ODN with Good Motifs

ODN_1	sequence (5'-3')	expt. 1	expt. 2	expt. 3
None			0.00	1.26
0.46				
1840	TCCATGTCGTTCCTGTCGTT	(SEQ ID NO:42)	2.33.	. .

This is the methylated version of ODN 1840; Z = 5-methyl cytosine LU is lytic units; ND = not done; **CpG** dinucleotides are underlined for clarity.

DETD Identification of Phosphorothioate ODN with Optimal **CPG** Motifs for Activation of Human B Cell Proliferation

DETD The ability of a **CpG** ODN to induce B cell proliferation is a good measure of its adjuvant potential. Indeed, ODN with strong adjuvant effects in mouse studies also induce B cell proliferation. To determine whether the optimal **CpG** ODN for inducing B cell proliferation are the same as those for inducing NK cell activity, similar panels of ODN (Table 6) were tested. Many **CpG** ODN were stimulatory. ODN 2006 produced the most consistant stimulation (Table 6).

DETD TABLE 6

Induction of human B cell proliferation by Phosphorothioate **CpG** ODN

DN	sequence (5'-3')	Stimulation Index[1] expt. 1	expt. 2	expt. 3	expt. 4	expt. 5	expt. 6
1840	TCCATGTCGTTCCTGTCGTT	(SEQ ID. . .					

DETD The ability of a **CpG** ODN to induce IL-12 secretion is a good measure of its adjuvant potential, especially in terms of its ability to. . . IL-12 secretion from human PBMC in vitro (Table 7) was examined. These experiments showed that in some human PBMC, most **CpG** ODN could induce IL-12 secretion (e.g., expt. 1). However, other donors responded to just a few **CpG** ODN (e.g., expt. 2). ODN 2006 was a consistent inducer of IL12 secretion from most subjects (Table 7).

DETD TABLE 7

Induction of human IL-12 secretion by Phosphorothioate **CpG** ODN

ODN_1	sequence (5'-3')	IL-12 (pg/ml) expt. 1	expt. 2
None		0	0
1962	TCCTGTCGTTCCTTGTCGTT	(SEQ ID NO:13) 19	0
1965	TCCTGTCGTTTTTTGTCGTT	(SEQ ID. . .	

DETD . . . DNA can directly activate highly purified B cells and monocytic cells. There are many similarities in the mechanism through which **CpG** DNA activates these cell types. For example, both require NFkB activation as explained further below.

DETD In further studies of different immune effects of **CpG** DNA, it was found that there is more than one type of **CpG** motif. Specifically, oligo 1668, with the best mouse B cell motif, is a strong inducer of both B cell and. . .

DETD TABLE 8

Different **CpG** motifs stimulate optimal murine B cell and NK activation

ODN	Sequence	B cell activation[1]	NK activation[2]
1668	TCCATGACGTTCCTGATGCT (SEQ ID NO:56)	42,849	2.52
1758	TCTCCCAGCGTGCGCCAT (SEQ ID NO:27)	1,747	6.66
NONE		367	0.00

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance.

[1] Measured by ^{3}H thymidine. . .

DETD . . . mice were then treated with oligonucleotides (30 μg in 200 μl saline by i.p. injection), which either contained an unmethylated **CpG** motif, i.e.,

DETD **TCCATGACGTTCCTGACGTT** (SEQ ID NO:39),

DETD . . . inflammatory cells are present in the lungs. However, when the mice are initially given a nucleic acid containing an unmethylated **CpG** motif along with the eggs, the inflammatory cells in the lung are not increased by subsequent inhalation of the egg. . .

DETD . . . inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a **CpG** oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes. . .

DETD FIG. 13 shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can actually redirect the cytokine response of the lung to production of Il12, indicating a Th1 type of immune. . .

DETD FIG. 14 shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune. .

. . . air flow results from endotoxin exposure, a therapeutically effective amount of a nucleic acid sequence containing at least one unmethylated **CpG**.

24. The method of claim 1 wherein the nucleic acid sequence containing at least one unmethylated **CpG** is administered by a route selected from the group consisting of intravenous, parenteral, oral, implant and topical.

. . . LPS, a therapeutically effective amount for inhibiting an inflammatory response of a nucleic acid sequence containing at least one unmethylated **CpG**.

. . . therapeutically effective amount for modifying the level of a cytokine of a nucleic acid sequence containing at least one unmethylated **CpG** dinucleotide.

L15 ANSWER 10 OF 12 USPATFULL on STN
2001:44204 **Immunostimulatory** nucleic acid molecules.
Krieg, Arthur M., Iowa City, IA, United States
Kline, Joel, Iowa City, IA, United States
Klinman, Dennis, Potomac, MD, United States
Steinberg, Alfred D., Potomac, MD, United States
University of Iowa Research Foundation, Iowa City, IA, United States (U.S. corporation)Coley Pharmaceutical Group, Inc., Wellesley, MA, United States (U.S. corporation)The United States of America as represented by the Department of Health and Human Services, Washington, DC, United States (U.S. government)
US 6207646 B1 20010327
APPLICATION: US 1996-738652 19961030 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. A method for ameliorating an immune system deficiency in a subject, comprising the steps of: a) contacting lymphocytes obtained from the subject with an antigen and an **immunostimulatory** nucleic acid, comprising: 5' X_1 CGX_2 3' wherein the **immunostimulatory** nucleic acid includes at least 8 nucleotides and wherein C is unmethylated, wherein X_1 and X_2 are nucleotides and wherein the **immunostimulatory** nucleic acid is an **immunostimulatory** nucleic acid selected from the group consisting of a synthetic **immunostimulatory** nucleic acid and an **immunostimulatory** nucleic acid having a phosphate modified backbone ex vivo, thereby producing activated lymphocytes; and b) readministering the activated lymphocytes obtained in step a) to the subject.

2. A method of claim 1, wherein the immune system deficiency is selected from the group consisting of: the presence of a tumor, cancer or infectious agent in the subject.

3. A method for desensitizing a subject against the occurrence of an allergic reaction in response to contact with a particular allergen, comprising administering to the subject an effective amount of an **immunostimulatory** nucleic acid, comprising: 5' X_1 CGX_2 3' wherein the **immunostimulatory** nucleic acid includes at least 8 nucleotides and wherein C is unmethylated and wherein X_1 and X_2 are nucleotides and an effective amount of the allergen.

4. A method of vaccination in a subject, comprising administering to the subject a vaccine antigen or an antigen encoded in a DNA vaccine and **immunostimulatory** nucleic acid, comprising: 5' X_1 CGX_2 3' wherein the **immunostimulatory** nucleic acid includes at least 8 nucleotides and wherein C is unmethylated and wherein X_1 and X_2 are nucleotides.

5. A method for treating leukemia in a subject, comprising administering to the subject an **immunostimulatory** nucleic acid, comprising: 5' X_1 CGX_2 3' wherein the **immunostimulatory** nucleic acid includes at least 8 nucleotides and wherein C is unmethylated, wherein X_1 and X_2 are nucleotides, prior to or in conjunction with a

chemotherapy, so that the subject's leukemia cells are more sensitive to the chemotherapy.

6. A composition comprising: a plasmid including an **immunostimulatory** nucleic acid sequence, comprising: 5'X_1 X_2 CGX_3 X_4 3' wherein C is unmethylated, wherein X_1, X_2, X_3 and X_4 are nucleotides and an antigen in a pharmaceutically acceptable carrier.

7. The composition of claim 6, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA and ApA; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, GpT, and TpG.

8. The composition of claim 6, further comprising a B-cell targeting molecule.

9. The composition of claim 8, wherein the targeting molecule is selected from the group consisting of a sterol, a lipid and a B-cell specific binding agent.

10. The composition of claim 6, wherein the antigen is encoded in a DNA vaccine.

11. The composition of claim 6, wherein the antigen is selected from the group consisting of proteins, polysaccharides, polysaccharide conjugates, glycolipids, viruses, bacteria, fungi, parasites, and allergens.

12. The composition of claim 11, wherein the antigen is an allergen.

13. The composition of claim 11, wherein the antigen is derived from an infectious organism selected from the group consisting of infectious bacteria, infectious virus, and infectious fungi.

14. A composition comprising: an isolated **immunostimulatory** nucleic acid of 8 to 100 nucleotides in length, comprising: 5' X_1 CGX_2 3' wherein C is unmethylated, wherein X_1 and X_2 are nucleotides, and an antigen in a pharmaceutically acceptable carrier.

15. The composition of claim 14, wherein the isolated **immunostimulatory** nucleic acid includes a phosphate backbone modification which is a phosphorothioate or phosphorodithioate modification.

16. The composition of claim 14, wherein the antigen is encoded in a DNA vaccine.

17. The composition of claim 14, wherein the antigen is selected from the group consisting of proteins, polysaccharides, polysaccharide conjugates, glycolipids, viruses, bacteria, fungi, parasites, and allergens.

18. A composition comprising: an **immunostimulatory** nucleic acid comprising: 5' X_1 CGX_2 3' wherein the **immunostimulatory** nucleic acid includes at least 8 nucleotides and wherein C is unmethylated, wherein X_1 and X_2 are nucleotides, and an antigen in a pharmaceutically acceptable carrier.

19. The composition of claim 18, wherein the synthetic **immunostimulatory** nucleic acid includes a phosphate backbone modification which is a phosphorothioate or phosphorodithioate modification.

20. The composition of claim 18, wherein the antigen is encoded in a DNA vaccine.

21. The composition of claim 18, wherein the antigen is selected from the group consisting of proteins, polysaccharides, polysaccharide conjugates, glycolipids, viruses, bacteria, fungi, parasites, and allergens.

22. A composition comprising: an **immunostimulatory** nucleic acid of 8 to 40 nucleotides in length, comprising: 5' X_1 CGX_2 3' wherein C is unmethylated, wherein X_1 and X_2 are nucleotides, and an antigen in a pharmaceutically acceptable carrier.

23. The composition of claim 22, wherein the **immunostimulatory** nucleic

acid includes a phosphate backbone modification which is a phosphorothioate or phosphorodithioate modification.

24. The composition of claim 22, wherein the antigen is encoded in a DNA vaccine.

25. The composition of claim 22, wherein the antigen is selected from the group consisting of proteins, polysaccharides, polysaccharide conjugates, glycolipids, viruses, bacteria, fungi, parasites, and allergens.

26. A method of inducing an antigen-specific immune response in a subject comprising: administering a vaccine to a subject, wherein the vaccine includes an antigen in combination with an **immunostimulatory** nucleic acid of claims 6, 14, 18 or 22 in an amount effective to induce an immune response.

27. The method of claim 26, wherein the antigen is selected from the group consisting of proteins, polysaccharides or polysaccharide conjugates, glycolipids, viruses, bacteria, fungi, parasites, and allergens.

28. The method of claim 27, wherein leukocytes of the subject are isolated and contacted with the antigen and **immunostimulatory** nucleic acid to produce activated leukocytes and wherein the activated leukocytes are readministered to the subject.

29. The method of claim 26, wherein the vaccine is administered ex vivo.

30. The composition of claim 18, wherein the **immunostimulatory** nucleic acid molecule is 8 to 100 nucleotides in length.

31. A composition comprising: an **immunostimulatory** nucleic acid, comprising: 5' X_1 X_2 CGX_3 X_4 3' wherein the **immunostimulatory** nucleic acid includes at least 8 nucleotides, wherein C is unmethylated, wherein X_1, X_2, X_3 and X_4 are nucleotides, and wherein at least one nucleotide has a phosphate backbone modification, and an antigen in a pharmaceutically acceptable carrier.

32. The composition of claim 31, wherein the **immunostimulatory** nucleic acid molecule is 8 to 100 nucleotides in length.

33. The composition of claim 31, wherein X_1 X_2 are dinucleotides selected from the group consisting of: GpT, GpG, GpA ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG.

34. The composition of claim 31, wherein X_3 X_4 are dinucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

35. The composition of claim 31, wherein the **immunostimulatory** nucleic acid is associated with a cationinc lipid.

36. The composition of claim 31, wherein X_1 X_2 are dinucleotides selected from the group consisting of GpT, GpG, GpA, and ApA and wherein X_3 X_4 are dinucleotides selected from the group consisting of TpT, CpT and ApT.

37. The composition of claim 31, wherein the antigen is selected from the group consisting of proteins, polysaccharides, polysaccharide conjugates, glycolipids, viruses, bacteria, fungi, parasites, and allergens.

38. The composition of claim 37, wherein the antigen is an allergen.

39. The composition of claim 37, wherein the antigen is derived from an infectious organism selected from the group consisting of infectious bacteria, infectious virus, and infectious fungi.

TI **Immunostimulatory** nucleic acid molecules
AI **US 1996-738652 19961030 (8)**
AB Nucleic acids containing unmethylated **CpG** dinucleotides and therapeutic utilities based on their ability to stimulate an immune response and to redirect a Th2 response to. . .
SUMM . . . cAMP response element, the CRE, the consensus form of which is the unmethylated sequence TGACGTC (binding is abolished if the **CpG** is methylated) (Iguchi-Ariga, S. M. M., and W. Schaffner: "**CpG** methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA

abolishes specific factor binding as well as transcriptional activation". Genes & Develop. 3:612,. . .

SUMM The instant invention is based on the finding that certain nucleic acids containing unmethylated cytosine-guanine (**CpG**) dinucleotides activate lymphocytes in a subject and redirect a subject's immune response from a Th2 to a Th1 (e.g. by. . . to produce Th1 cytokines, including IL-12, IFN-γ and GM-CSF). Based on this finding, the invention features, in one aspect, novel **immunostimulatory** nucleic acid compositions.

SUMM In a preferred embodiment, the **immunostimulatory** nucleic acid contains a consensus mitogenic **CpG** motif represented by the formula:

SUMM In a particularly preferred embodiment an **immunostimulatory** nucleic acid molecule contains a consensus mitogenic **CpG** motif represented by the formula:

SUMM Enhanced **immunostimulatory** activity of human cells occurs where X_1 X_2 is selected from the group consisting of GpT, GpG, GpA and ApA. . . X_3 X_4 is selected from the group consisting of TpT, CpT and GpT (Table 5). For facilitating uptake into cells, **CpG** containing **immunostimulatory** nucleic acid molecules are preferably in the range of 8 to 40 base pairs in size. However, nucleic acids of any size (even many kb long) are **immunostimulatory** if sufficient **immunostimulatory** motifs are present, since such larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not include a GCG trinucleotide sequence at or near the 5' and/or 3' terminals and/or the consensus mitogenic **CpG** motif is not a palindrome. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, particularly phosphorothioate stabilized oligonucleotides.

SUMM In a second aspect, the invention features useful therapies, which are based on the **immunostimulatory** activity of the nucleic acid molecules. For example, the **immunostimulatory** nucleic acid molecules can be used to treat, prevent or ameliorate an immune system deficiency (e.g., a tumor or cancer or a viral, fungal, bacterial or parasitic infection in a subject). In addition, **immunostimulatory** nucleic acid molecules can be administered to stimulate a subject's response to a vaccine.

SUMM Further, the ability of **immunostimulatory** nucleic acid molecules to induce leukemic cells to enter the cell cycle supports the use of **immunostimulatory** nucleic acid molecules in treating leukemia by increasing the sensitivity of chronic leukemia cells and then administering conventional ablative chemotherapy, or combining the **immunostimulatory** nucleic acid molecules with another immunotherapy.

DRWD . . . and calf thymus DNA) (.diamond-solid.). B. Control phosphodiester oligodeoxynucleotide (ODN) 5' ATGGAAGGTCCAGTGTTCTC3' (SEQ ID No: 1) (.box-solid.) and two phosphodiester **CpG** ODN 5' ATCGACCTACGTGCGTTCTC3' (SEQ ID No: 2) (.diamond-solid.) and 5' TCCATAACGTTCCTGATGCT3' (SEQ ID No: 3) (.circle-solid.) C. Control phosphorothioate ODN 5' GCTAGATGTTAGCGT3' (SEQ ID No: 4) (.box-solid.) and two phosphorothioate **CpG** ODN 5' GAGAACGTCGACCTTCGAT3' (SEQ ID No: 5) (.diamond-solid.) and 5' GCATGACGTTGAGCT3' (SEQ ID No: 6) (.circle-solid.). Data present the mean±standard. . .

DRWD FIG. 2 is a graph plotting IL-6 production induced by **CpG** DNA in vivo as determined 1-8 hrs after injection. Data represent the mean from duplicate analyses of sera from two mice. BALB/c mice (two mice/group) were injected iv. with 100 μl of PBS (.quadrature.) or 200 μg of **CpG** phosphorothioate ODN 5' TCCATGACGTTCCTGATGCT3' (SEQ ID No: 7) (.box-solid.) or non-**CpG** phosphorothioate ODN 5 'TCCATGAGCTTCCTGAGTCT3' (SEQ ID No: 8) (.diamond-solid.).

DRWD . . . periods after in vivo stimulation of BALB/c mice (two mice/group) injected iv with 100 μl of PBS, 200 μg of **CpG** phosphorothioate ODN 5' TCCATGACGTTCCTGATGCT3' (SEQ ID No: 7) or non-**CpG** phosphorothioate ODN 5' TCCATGAGCTTCCTGAGTCT3' (SEQ ID No: 8).

DRWD FIG. 4A is a graph plotting dose-dependent inhibition of **CpG**-induced IgM production by anti-IL-6. Splenic B-cells from DBA/2 mice were stimulated with **CpG** ODN 5' TCCAAGACGTTCCTGATGCT3' (SEQ ID No: 9) in the presence of the indicated concentrations of neutralizing anti-IL-6 (.diamond-solid.) or isotype control Ab (.circle-solid.) and IgM levels in culture supernatants determined by ELISA. In the absence of **CpG** ODN, the anti-IL-6 Ab had no effect on IgM secretion (.box-solid.).

DRWD FIG. 4B is a graph plotting the stimulation index of **CpG**-induced splenic B cells cultured with anti-IL-6 and **CpG** S-ODN 5' TCCATGACGTTCCTGATGCT3' (SEQ ID No: 7) (.diamond-solid.) or anti-IL-6 antibody only (.box-solid.). Data present the mean±standard deviation of. . .

DRWD . . . cells transfected with a promoter-less CAT construct (pCAT), positive control plasmid (RSV), or IL-6 promoter-CAT construct alone or cultured with **CpG** 5' TCCATGACGTTCCTGATGCT3' (SEQ ID No: 7) or non-**CpG** 5' TCCATGAGCTTCCTGAGTCT3' (SEQ ID No: 8)

phosphorothioate ODN at the indicated concentrations. Data present the mean of triplicates.

DRWD FIG. 6 is a schematic overview of the immune effects of the **immunostimulatory** unmethylated **CpG** containing nucleic acids, which can directly activate both B cells and monocytic cells (including macrophages and dendritic cells) as shown. The **immunostimulatory** oligonucleotides do not directly activate purified NK cells, but render them competent to respond to IL-12 with a marked increase in their IFN-γ production. By inducing IL-12 production and the subsequent increased IFN-γ secretion by NK cells, the **immunostimulatory** nucleic acids promote a Th1 type immune response. No direct activation of proliferation of cytokine secretion by highly purified T cells has been found. However, the induction of Th1 cytokine secretion by the **immunostimulatory** oligonucleotides promotes the development of a cytotoxic lymphocyte response.

DRWD FIG. 7 is an autoradiograph showing NFκB mRNA induction in monocytes treated with E. coli (EC) DNA (containing unmethylated **CpG** motifs), control (CT) DNA (containing no unmethylated **CpG** motifs) and lipopolysaccharide (LPS) at various measured times, 15 and 30 minutes after contact.

DRWD . . . the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the **CpG** oligo (**TCCATGACGTTCCTGACGTT** SEQ ID No. 10) also showed an increase in the level of reactive oxygen species such that more than 50%. . .

DRWD . . . have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with **CpG** DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and. . .

DRWD . . . egg antigen "SEA" (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given **CpG** oligo (SEQ ID NO. 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation. . .

DRWD . . . and subsequently inhale SEA (open circle), many eosinophils are present in the lungs. However, when the mice are initially given **CpG** oligo (SEQ ID NO. 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation. . .

DRWD . . . inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a **CpG** oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes. . .

DRWD . . . or SEQ ID No. 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can actually redirect the cytokine response of the lung to production of IL-12, indicating a Th1 type of immune. . .

DRWD . . . or SEQ ID No. 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune. . .

DETD An "**immunostimulatory** nucleic acid molecule" refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (i.e. "**CpG** DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates (e.g. has a mitogenic effect on, or induces or increases cytokine expression by) a vertebrate lymphocyte. An **immunostimulatory** nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have. . .

DETD In a preferred embodiment, the **immunostimulatory** nucleic acid contains a consensus mitogenic **CpG** motif represented by the formula:

DETD In a particularly preferred embodiment, **immunostimulatory** nucleic acid molecules are between 2 to 100 base pairs in size and contain a consensus mitogenic **CpG** motif represented by the formula:

DETD For economic reasons, preferably the **immunostimulatory CpG** DNA is in the range of between 8 to 40 base pairs in size if it is synthesized as an oligonucleotide. Alternatively, **CpG** dinucleotides can be produced on a large scale in plasmids, which after being administered to a subject are degraded into oligonucleotides. Preferred **immunostimulatory** nucleic acid molecules (e.g. for use in increasing the effectiveness of a vaccine or to treat an immune system deficiency. . .

DETD The stimulation index of a particular **immunostimulatory CpG** DNA can be tested in various immune cell assays. Preferably, the stimulation index of the **immunostimulatory CpG** DNA with regard to B-cell proliferation is at least about 5, preferably at least about 10, more preferably at least. . . treat an immune system deficiency by stimulating a cell-mediated (local) immune response in a subject, it is important that the **immunostimulatory CpG** DNA be capable of effectively inducing cytokine secretion by monocytic cells and/or Natural Killer (NK) cell lytic activity.

DETD Preferred **immunostimulatory CpG** nucleic acids should effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml. . . IL-6, 200 pg/ml IL-12, depending on the therapeutic indication, as determined by the assays described in Example 12. Other preferred **immunostimulatory CpG** DNAs should effect at least about 10%, more preferably at least about 15% and most preferably at least about 20%. . .

DETD . . . in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated **CpG** containing nucleic acid molecules that are tens to hundreds of kbs long are relatively resistant to in vivo degradation. For shorter **immunostimulatory** nucleic acid molecules, secondary structure can stabilize and increase their effect. For example, if the 3' end of a nucleic. . .

DETD . . . acid molecules (including phosphorodithioate-modified) can increase the extent of immune stimulation of the nucleic acid molecule, which contains an unmethylated **CpG** dinucleotide as shown herein. International Patent Application Publication Number: WO 95/26204 entitled "Immune Stimulation By Phosphorothioate Oligonucleotide Analogs" also reports on the non-sequence specific **immunostimulatory** effect of phosphorothioate modified oligonucleotides. As reported herein, unmethylated **CpG** containing nucleic acid molecules having a phosphorothioate backbone have been found to preferentially activate B-cell activity, while unmethylated **CpG** containing nucleic acid molecules having a phosphodiester backbone have been found to preferentially activate monocytic (macrophages, dendritic cells and monocytes) and NK cells. Phosphorothioate **CpG** oligonucleotides with preferred human motifs are also strong activators of monocytic and NK cells.

DETD Certain Unmethylated **CpG** Containing Nucleic Acids Have B Cell Stimulatory Activity As Shown in vitro and in vivo

DETD . . . the other nonstimulatory control ODN. In comparing these sequences, it was discovered that all of the four stimulatory ODN contained **CpG** dinucleotides that were in a different sequence context from the nonstimulatory control.

DETD To determine whether the **CpG** motif present in the stimulatory ODN was responsible for the observed stimulation, over 300 ODN ranging in length from 5 to 42 bases that contained methylated, unmethylated, or no **CpG** dinucleotides in various sequence contexts were synthesized. These ODNs, including the two original "controls" (ODN 1 and 2) and two. . . then examined for in vitro effects on spleen cells (representative sequences are listed in Table 1). Several ODN that contained **CpG** dinucleotides induced B cell activation and IgM secretion; the magnitude of this stimulation typically could be increased by adding more **CpG** dinucleotides (Table 1; compare ODN 2 to 2a or 3D to 3Da and 3Db). Stimulation did not appear to result. . .

DETD Mitogenic ODN sequences uniformly became nonstimulatory if the **CpG** dinucleotide was mutated (Table 1; compare ODN 1 to 1a; 3D to 3Dc; 3M to 3Ma; and 4 to 4a) or if the cytosine of the **CpG** dinucleotide was replaced by 5-methylcytosine (Table 1; ODN 1b,2b,3Dd, and 3Mb). Partial methylation of **CpG** motifs caused a partial loss of stimulatory effect (compare 2a to 2c, Table 1). In contrast, methylation of other cytosines did not reduce ODN activity (ODN 1c, 2d, 3De and 3Mc). These data confirmed that a **CpG** motif is the essential element present in ODN that activate B cells.

DETD In the course of these studies, it became clear that the bases flanking the **CpG** dinucleotide played an important role in determining the murine B cell activation induced by an ODN. The optimal stimulatory motif was determined to consist of a **CpG** flanked by two 5' purines (preferably a GpA dinucleotide) and two 3' pyrimidines (preferably a TpT or TpC dinucleotide). Mutations of ODN to bring the **CpG** motif closer to this ideal improved stimulation (e.g. Table 1, compare ODN 2 to 2e; 3M to 3Md) while mutations. . . Table 1, compare ODN 3D to 3Df; 4 to 4b, 4c and 4d). On the other hand, mutations outside the **CpG** motif did not reduce stimulation (e.g. Table 1, compare ODN 1 to 1d; 3D to 3Dg; 3M to 3Me). For. . .

DETD . . . 10 As. The effect of the G-rich ends is cis; addition of an ODN with poly G ends but no **CpG** motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated **CpG** were found to be more **immunostimulatory**.

DETD . . . dev. derived from at least 3 separate experiments, and are compared to wells cultured with no added ODN.

ND = not done.

CpG dinucleotides are underlined.

Dots indicate identity; dashes indicate deletions.

Z indicates 5 methyl cytosine.

DETD TABLE 2

Identification of the optimal **CpG** motif for Murine IL-6 production and B cell

activation.

ODN (ng/ml)[c]	SEQUENCE (5'-3')	CH12.LX SI[b]	IL-6 (pg/ml)[a] SPLENIC B CELL IgM
512. . .		0.2	3534 ± 217
1708	(SEQ ID No:47)	.....CA..TG.....	ND
59 ± 3		1.5 ± 0.1	466 ± 109

Dots indicate identity; **CpG** dinucleotides are underlined; ND = not done

[a] The experiment was done at least three times with similar results. The level. . . CH12.LX and splenic B cells was ≤ 10 pg/ml. The IgM level of unstimulated culture was 547 ± 82 ng/ml. **CpG** dinucleotides are underlined and dots indicate identity.

[b] [3 H] Uridine uptake was indicated as a fold increase (SI: stimulation index) from unstimulated control (2322.67 ± 213.68 cpm). Cells were stimulated with 20 μM of various **CpG** O-QDN. Data present the mean ± SD of triplicates

[c] Measured by ELISA.

DETD . . . the subsequent fall in stimulation when purified B cells with or without anti-IgM (at a submitogenic dose) were cultured with **CpG** ODN, proliferation was found to synergistically increase about 10-fold by the two mitogens in combination after 48 hours. The magnitude. . .

DETD Cell cycle analysis was used to determine the proportion of B cells activated by **CpG**-ODN. **CpG**-ODN induced cycling in more than 95% of B cells. Splenic B lymphocytes sorted by flow cytometry into CD23- (marginal zone). . . as were both resting and activated populations of B cells isolated by fractionation over Percoll gradients. These studies demonstrated that **CpG**-ODN induce essentially all B cells to enter the cell cycle.

DETD **Immunostimulatory** Nucleic Acid Molecules Block Murine B Cell Apoptosis

DETD . . . are rescued from this growth arrest by certain stimuli such as LPS and by the CD40 ligand. ODN containing the **CpG** motif were also found to protect WEHI-231 from anti-IgM induced growth arrest, indicating that accessory cell populations are not required for the effect. Subsequent work indicates that **CpG** ODN induce Bcl-x and myc expression, which may account for the protection from apoptosis. Also, **CpG** nucleic acids have been found to block apoptosis in human cells. This inhibition of apoptosis is important, since it should enhance and prolong immune activation by **CpG** DNA.

DETD Induction of Murine Cytokine Secretion by **CpG** Motifs in Bacterial DNA or Oligonucleotides.

DETD As described in Example 9, the amount of IL-6 secreted by spleen cells after **CpG** DNA stimulation was measured by ELISA. T cell depleted spleen cell cultures rather than whole spleen cells were used for in vitro studies following preliminary studies showing that T cells contribute little or nothing to the IL-6 produced by **CpG** DNA-stimulated spleen cells. As shown in Table 3, IL-6 production was markedly increased in cells cultured with E. coli DNA. . . response to bacterial DNA. To analyze whether the IL-6 secretion induced by E. coli DNA was mediated by the unmethylated **CpG** dinucleotides in bacterial DNA, methylated E. coli DNA and a panel of synthetic ODN were examined. As shown in Table 3, **CpG** ODN significantly induced IL-6 secretion (ODN 5a, 5b, 5c) while **CpG** methylated E. coli DNA, or ODN containing methylated **CpG** (ODN 5f) or no **CpG** (ODN 5d) did not. Changes at sites other than **CpG** dinucleotides (ODN 5b) or methylation of other cytosines (ODN 5g) did not reduce the effect of **CpG** ODN. Methylation of a single **CpG** in an ODN with three CpGs resulted in a partial reduction in the stimulation (compare ODN 5c to 5e; Table. . .

DETD TABLE 3

Induction of Murine IL-6 secretion by **CpG** motifs in bacterial DNA or oligonucleotides.

Treatment	IL-6 (pg/ml)
calf thymus DNA	≤10
calf thymus DNA + DNase	≤10
E. coli DNA	1169.5 ± 94.1
E. coli DNA + DNase	≤10
CpG methylated E. coli DNA	≤10
LPS	280.1 ± 17.1
Media (no DNA)	≤10
ODN 5a SEQ. ID. ATGGACTCTCCAGCGTTCTC	1096.4 ± 372.0

. . .

DETD . . . μg/ml) with or without enzyme treatment, or LPS (10 μg/ml) for 24 hr. Data represent the mean (pg/ml)±SD of triplicates. **CpG** dinucleotides are underlined and dots indicate identity. Z indicates 5-methylcytosine.

DETD Identification of the optimal **CpG** motif for induction of Murine IL-6 and IgM secretion and B cell proliferation.

DETD To evaluate whether the optimal B cell stimulatory **CpG** motif was identical with the optimal **CpG** motif for IL-6 secretion, a panel of ODN in which the bases flanking the **CpG** dinucleotide were

progressively substituted was studied. This ODN panel was analyzed for effects on B cell proliferation, Ig production, and. . . splenic B cells and CH12.LX cells. As shown in Table 2, the optimal stimulatory motif is composed of an unmethylated **CpG** flanked by two 5' purines and two 3' pyrimidines. Generally a mutation of either 5' purine to pyrimidine or 3'. . . had less marked effects. Based on analyses of these and scores of other ODN, it was determined that the optimal **CpG** motif for induction of IL-6 secretion is TGACGTT, which is identical with the optimal mitogenic and IgM-inducing **CpG** motif (Table 2). This motif was more stimulatory than any of the palindrome containing sequences studied (1639, 1707 and 1708).

DETD Titration of Induction of Murine IL-6 Secretion by **CpG** Motifs.

DETD Bacterial DNA and **CpG** ODN induced IL-6 production in T cell depleted murine spleen cells in a dose-dependent manner, but vertebrate DNA and non-**CpG** ODN did not (FIG. 1). IL-6 production plateaued at approximately 50 μg/ml of bacterial DNA or 40 μM of **CpG** O-ODN. The maximum levels of IL-6 induced by bacterial DNA and **CpG** ODN were 1-1.5 ng/ml and 2-4 ng/ml respectively. These levels were significantly greater than those seen after stimulation by LPS (0.35 ng/ml) (FIG. 1A). To evaluate whether **CpG** ODN with a nuclease-resistant DNA backbone would also induce IL-6 production, S-ODN were added to T cell depleted murine spleen cells. **CpG** S-ODN also induced IL-6 production in a dose-dependent manner to approximately the same level as **CpG** O-ODN while non-**CpG** S-ODN failed to induce IL-6 (FIG. 1C). **CpG** S-ODN at a concentration of 0.05 μM could induce maximal IL-6 production in these cells. This result indicated that the nuclease-resistant DNA backbone modification retains the sequence specific ability of **CpG** DNA to induce IL-6 secretion and that **CpG** S-ODN are more than 80-fold more potent than **CpG** O-ODN in this assay system.

DETD Induction of Murine IL-6 Secretion by **CpG** DNA In Vivo.

DETD To evaluate the ability of bacterial DNA and **CpG** S-ODN to induce IL-6 secretion in vivo, BALB/c mice were injected iv. with 100 μg of E. coli DNA, calf thymus DNA, or **CpG** or non-stimulatory S-ODN and bled 2 hr after stimulation. The level of IL-6 in the sera from the E. coli. . . 13 ng/ml while IL-6 was not detected in the sera from calf thymus DNA or PBS injected groups (Table 4). **CpG** S-ODN also induced IL-6 secretion in vivo. The IL-6 level in the sera from **CpG** S-ODN injected groups was approximately 20 ng/ml. In contrast, IL-6 was not detected in the sera from non-stimulatory S-ODN stimulated. . .

DETD TABLE 4

Secretion of Murine IL-6 induced by **CpG** DNA stimulation in vivo.

Stimulant	IL-6 (pg/ml)
PBS	<50
E coli DNA	13858 ± 3143
Calf Thymus DNA	<50
CpG S-ODN	20715 ± 606
non-**CpG** S-ODN	<50

DETD . . . injected with 100 μl of PBS, 200 μg of E. coli DNA or calf thymus DNA, or 500 μg of **CpG** S-ODN or non-**CpG** control S-ODN. Mice were bled 2 hr after injection and 1:10 dilution of each serum was analyzed by IL-6 ELISA. Sensitivity limit of IL-6 ELISA was 5 pg/ml. Sequences of the **CpG** S-ODN is 5'GCATGACGTTGAGCT3' (SEQ. ID. No: 48) and of the non-stimulatory S-ODN is 5'GCTAGATGTTAGCGT3' (SEQ. ID. No: 49). Note that although there is a **CpG** in sequence 48, it is too close to the 3' end to effect stimulation, as explained herein. Data represent mean±SD. . .

DETD Kinetics of Murine IL-6 secretion after stimulation by **CpG** motifs in vivo.

DETD To evaluate the kinetics of induction of IL-6 secretion by **CpG** DNA in vivo, BALB/c mice were injected iv. with **CpG** or control non-**CpG** S-ODN. Serum IL-6 levels were significantly increased within 1 hr and peaked at 2 hr to a level of approximately 9 ng/ml in the **CpG** S-ODN injected group (FIG. 2). IL-6 protein in sera rapidly decreased after 4 hr and returned to basal level by 12 hr after stimulation. In contrast to **CpG** DNA -stimulated groups, no significant increase of IL-6 was observed in the sera from the non-stimulatory S-ODN or PBS injected. .

DETD Tissue distribution and kinetics of IL-6 mRNA expression induced by **CpG** motifs in vivo.

DETD As shown in FIG. 2, the level of serum IL-6 increased rapidly after **CpG** DNA stimulation. To investigate the possible tissue origin of this serum IL-6, and the kinetics of IL-6 gene expression in vivo after **CpG** DNA stimulation, BALB/c mice were injected iv with **CpG** or non-**CpG** S-ODN and RNA was extracted from liver, spleen, thymus, and bone marrow at various time points after stimulation. As shown. . . FIG. 3A, the level of IL-6 mRNA in liver, spleen, and thymus was increased within 30 min. after injection of **CpG** S-ODN. The liver IL-6 mRNA peaked at 2 hr post-injection and rapidly decreased and reached basal level 8 hr after. . . hr post-injection and then gradually decreased (FIG. 3A). IL-6

mRNA was significantly increased in bone marrow within 1 hr after **CpG** S-ODN injection but then returned to basal level. In response to **CpG** S-ODN, liver, spleen and thymus showed more substantial increases in IL-6 mRNA expression than the bone marrow.

DETD Patterns of Murine Cytokine Expression Induced by **CpG** DNA

DETD . . . within 30 minutes and the level of IL-6 increased strikingly within 2 hours in the serum of mice injected with **CpG** ODN. Increased expression of IL-12 and interferon gamma (IFN-γ) mRNA by spleen cells was also detected within the first two. . .

DETD TABLE 5

Induction of human PBMC cytokine secrtetion by **CpG** oligos

ODN	Sequence (5'-3')	IL-6[1]	TNF-α[1]	IFN-γ[1]	GM-CSF	IL-12
512	TCCATGTCGGTCCTGATGCT	500	140	15.6	70	250
SEQ ID NO:37						
1637	......C.....	550	16	7.8.	. .	ID NO:45
1707	......A..TC.....	300	70	17	0	0
SEQ ID NO:46						
1708	.....CA..TG.....	270	10	17	0	0
SEQ ID NO:47						

dots indicate identity; **CpG** dinucleotides are underlined

[1] measured by ELISA using Quantikine kits from R&D Systems (pg/ml) Cells were cultured in 10% autologous serum. . .

DETD **CpG** DNA Induces Cytokine Secretion by Human PBMC, Specifically Monocytes

DETD . . . panels of ODN used for studying mouse cytokine expression were used to determine whether human cells also are induced by **CpG** motifs to express cytokine (or proliferate), and to identify the **CpG** motif(s) responsible. Oligonucleotide 1619 (GTCGTT) was the best inducer of TNF-α and IFN-γ secretion, and was closely followed by a. . . little induction of other cytokines. Thus, it appears that human lymphocytes, like murine lymphocytes, secrete cytokines differentially in response to **CpG** dinucleotides, depending on the surrounding bases. Moreover, the motifs that stimulate murine cells best differ from those that are most effective with human cells. Certain **CpG** oligodeoxynucleotides are poor at activating human cells (oligodeoxynucleotides 1707, 1708, which contain the palindrome forming sequences GACGTC and CACGTG respectively).

DETD . . . simply reflect a nonspecific death all all cell types. Cytokine secretion in response to E. coli (EC) DNA requires unmethylated **CpG** motifs, since it is abolished by methylation of the EC DNA (next to the bottom row, Table 6). LPS contamination. . .

DETD TABLE 6

CpG DNA induces cytokine secretion by human PBMC

DNA	TNF-α (pg/ml)[1]	IL-6 (pg/ml)	IFN-γ (pg/ml)	RANTES (pg/ml)
EC DNA (50 μg/ml)	900	12,000.	. .	cytokine production under these

conditions was from monocytes (or other L-LME-sensitive cells).

[3] EC DNA was methylated using 2U/μg DNA of **CpG** methylase (New England Biolabs) according to the manufacturer's directions, and methylation confirmed by digestion with Hpa-II and Msp-I. As a. . .

DETD . . . cytokine production in the PBMC treated with L-LME suggested, that monocytes may be responsible for cytokine production in response to **CpG** DNA. To test this hypothesis more directly, the effects of **CpG** DNA on highly purified human monocytes and macrophages was tested. As hypothesized, **CpG** DNA directly activated production of the cytokines IL-6, GM-CSF, and TNF-α by human macrophages, whereas non-**CpG** DNA did not (Table 7).

DETD TABLE 7

CpG DNA induces cytokine expression in purified human macrophages

	IL-6 (pg/ml)	GM-CSF (pg/ml)	TNF-α (pg/ml)
Cells alone	0	0	0
CT DNA (50. . .			

DETD Biological Role of IL-6 in Inducing Murine IgM Production in Response to **CpG** Motifs.

DETD The kinetic studies described above revealed that induction of IL-6 secretion, which occurs within 1 hr post **CpG** stimulation, precedes IgM secretion. Since the optimal **CpG** motif for ODN inducing secretion of IL-6 is the same as that for IgM (Table 2), whether the **CpG** motifs independently induce IgM and IL-6 production or whether the IgM production is dependent on prior IL-6 secretion was examined. The addition of neutralizing anti-IL-6 antibodies inhibited in vitro IgM production mediated by **CpG** ODN in a dose-dependent manner but a control antibody did not (FIG. 4A). In contrast, anti-IL-6 addition did not affect either the basal level or the **CpG**-induced B cell proliferation (FIG. 4B).

DETD Increased transcriptional activity of the IL-6 promoter in response to **CpG** DNA.

DETD The increased level of IL-6 mRNA and protein after **CpG** DNA stimulation could result from transcriptional or post-transcriptional regulation. To

determine if the transcriptional activity of the IL-6 promoter was upregulated in B cells cultured with **CpG** ODN, a murine B cell line, WEHI-231, which produces IL-6 in response to **CpG** DNA, was transfected with an IL-6 promoter-CAT construct (pIL-6/CAT) (Pottratz, S. T. et al., 17B-estradiol) inhibits expression of human interleukin-6-promoter-reporter constructs by a receptor-dependent mechanism. J. Clin. Invest. 93:944). CAT assays were performed after stimulation with various concentrations of **CpG** or non-**CpG** ODN. As shown in FIG. 5, **CpG** ODN induced increased CAT activity in dose-dependent manner while non-**CpG** ODN failed to induce CAT activity. This confirms that **CpG** induces the transcriptional activity of the IL-6 promoter.

DETD Dependence of B cell activation by **CpG** ODN on the Number of 5' and 3' Phosphorothioate Internucleotide Linkages.

DETD . . . DNA synthesis (by ^{3}H thymidine incorporation) in treated spleen cell cultures (Example 10). O-ODN (0/0 phosphorothioate modifications) bearing a **CpG** motif caused no spleen cell stimulation unless added to the cultures at concentrations of at least 10 μM (Example 10).. . .

DETD Dependence of **CpG**-Mediated Lymphocyte Activation on the Type of Backbone Modification.

DETD . . . result from the nuclease resistance of the former. To determine the role of ODN nuclease resistance in immune stimulation by **CpG** ODN, the stimulatory effects of chimeric ODN in which the 5' and 3' ends were rendered nuclease resistant with either. . .

DETD . . . while the S-ODN with the 3D sequence was less potent than the corresponding S-O-ODN (Example 10). In comparing the stimulatory **CpG** motifs of these two sequences, it was noted that the 3D sequence is a perfect match for the stimulatory motif in that the **CpG** is flanked by two 5' purines and two 3' pyrimidines. However, the bases immediately flanking the **CpG** in ODN 3D are not optimal; it has a 5' pyrimidine and a 3' purine. Based on further testing, it. . . for immune stimulation is more stringent for S-ODN than for S-O- or O-ODN. S-ODN with poor matches to the optimal **CpG** motif cause little or no lymphocyte activation (e.g. Sequence 3D). However, S-ODN with good matches to the motif, most critically at the positions immediately flanking the **CpG**, are more potent than the corresponding S-O-ODN (e.g. Sequence 3M, Sequences 4 and 6), even though at higher concentrations (greater. . .

DETD The increased B cell stimulation seen with **CpG** ODN bearing S or S_2 substitutions could result from any or all of the following effects: nuclease resistance, increased cellular. . . However, nuclease resistance can not be the only explanation, since the MP-O-ODN were actually less stimulatory than the O-ODN with **CpG** motifs. Prior studies have shown that ODN uptake by lymphocytes is markedly affected by the backbone chemistry (Zhao et al.,. . .

DETD Unmethylated **CpG** Containing Oligos Have NK Cell Stimulatory Activity

DETD Experiments were conducted to determine whether **CpG** containing oligonucleotides stimulated the activity of natural killer (NK) cells in addition to B cells. As shown in Table 8, a marked induction of NK activity among spleen cells cultured with **CpG** ODN 1 and 3Dd was observed. In contrast, there was relatively no induction in effectors that had been treated with non-**CpG** control ODN.

DETD TABLE 8

Induction Of NK Activity By **CpG** Oligodeoxynucleotides (ODN)

	% YAC-1 Specific Lysis*		% 2C11 Specific Lysis	
	Effector: Target		Effector: Target	
ODN	50:1	100:1	50:1	100:1
None	-1.1 -1.4	15.3	16.6	
1	16.1	24.5	38.7	47.2
3Dd	17.1	27.0	37.0	40.0
non-**CpG** ODN	-1.6 -1.7	14.8	15.4	

DETD Induction of NK activity by DNA containing **CpG** motifs, but not by non-**CpG** DNA.

DETD . . . 9). To determine whether the stimulatory activity of bacterial DNA may be a consequence of its increased level of unmethylated **CpG** dinucleotides, the activating properties of more than 50 synthetic ODN containing unmethylated, methylated, or no **CpG** dinucleotides was tested. The results, summarized in Table 9, demonstrate that synthetic ODN can stimulate significant NK activity, as long as they contain at least one unmethylated **CpG** dinucleotide. No difference was observed in the stimulatory effects of ODN in which the **CpG** was within a palindrome (such as ODN 1585, which contains the palindrome AACGTT) from those ODN without palindromes (such as 1613 or 1619), with the caveat that optimal stimulation was generally seen with ODN in which the **CpG** was flanked by two 5' purines or a 5' GpT dinucleotide and two 3' pyrimidines. Kinetic experiments demonstrated that NK. . . of the ODN. The data indicates that the murine NK response is dependent on the prior activation of monocytes by **CpG** DNA, leading to the production of IL-12, TNF-α, and IFN-α/β (Example 11).

DETD TABLE 9

Induction of NK Activity by DNA Containing **CpG** Motifs but not by Non-**CpG** DNA

DNA or Cytokine Added	LU/10^6 Mouse Cells	Human cells
Expt. 1 None	0.00	0.00
IL-2	16.68	15.82
E.Coli. DNA	7.23. . .	

DETD **CpG** dinucleotides in ODN sequences are indicated by underlying; Z indicates methylcytosine. Lower case letters indicate nuclease resistant phosphorothioate modified internucleotide. . .

DETD From all of these studies, a more complete understanding of the immune effects of **CpG** DNA has been developed, which is summarized in FIG. 6.

DETD As shown in FIG. 6, **CpG** DNA can directly activate highly purified B cells and monocytic cells. There are many similarities in the mechanism through which **CpG** DNA activates these cell types. For example, both require NFκB activation as explained further below.

DETD In further studies of different immune effects of **CpG** DNA, it was found that there is more than one type of **CpG** motif. Specifically, oligo 1668, with the best mouse B cell motif, is a strong inducer of both B cell and. . .

DETD TABLE 10

Different **CpG** motifs stimulate optimal murine B cell and NK activation

ODN	Sequence		B cell activation[1]	NK activation[2]
1668	TCCATGACGTTCCTGATGCT	(SEQ.ID.NO:54)	42,849	2.52
1758	TCTCCCAGCGTGCGCCAT	(SEQ.ID.NO.55)	1,747	6.66
NONE			367	0.00

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance.

[1] Measured by ^{3}H thymidine. . .

DETD Teleological Basis of **Immunostimulatory**, Nucleic Acids

DETD Vertebrate DNA is highly methylated and **CpG** dinucleotides are underrepresented. However, the stimulatory **CpG** motif is common in microbial genomic DNA, but quite rare in vertebrate DNA. In addition, bacterial DNA has been reported. . . P. et al., J. Immunol. 147:1759 (1991)). Experiments further described in Example 3, in which methylation of bacterial DNA with **CpG** methylase was found to abolish mitogenicity, demonstrates that the difference in **CpG** status is the cause of B cell stimulation by bacterial DNA. This data supports the following conclusion: that unmethylated **CpG** dinucleotides present within bacterial DNA are responsible for the stimulatory effects of bacterial DNA.

DETD Teleologically, it appears likely that lymphocyte activation by the **CpG** motif represents an immune defense mechanism that can thereby distinguish bacterial from host DNA. Host DNA, which would commonly be. . . regions and areas of inflammation due to apoptosis (cell death), would generally induce little or no lymphocyte activation due to **CpG** suppression and methylation. However, the presence of bacterial DNA containing unmethylated **CpG** motifs can cause lymphocyte activation precisely in infected anatomic regions, where it is beneficial. This novel activation pathway provides a rapid alternative to T cell dependent antigen specific B cell activation. Since the **CpG** pathway synergizes with B cell activation through the antigen receptor, B cells bearing antigen receptor specific for bacterial antigens would. . .

DETD . . . 35:647 (1992)), is likely triggered at least in part by activation of DNA-specific B cells through stimulatory signals provided by **CpG** motifs, as well as by binding of bacterial DNA to antigen receptors.

DETD . . . products released from dying bacteria that reach concentrations sufficient to directly activate many lymphocytes. Further evidence of the role of **CpG** DNA in the sepsis syndrome is described in Cowdery, J., et. al., (1996) The Journal of Immunology 156:4570-4575.

DETD Unlike antigens that trigger B cells through their surface Ig receptor, **CpG**-ODN did not induce any detectable Ca^{2+} flux, changes in protein tyrosine phosphorylation, or IP 3 generation. Flow cytometry with FITC-conjugated ODN with or without a **CpG** motif was performed as described in Zhao, Q et al., (Antisense Research and Development 3:53-66 (1993)), and showed equivalent membrane binding, cellular uptake, efflux, and intracellular localization. This suggests that there may not be cell membrane proteins specific for **CpG** ODN. Rather than acting through the cell membrane, that data suggests that unmethylated **CpG** containing oligonucleotides require cell uptake for activity: ODN covalently linked to a solid Teflon support were nonstimulatory, as were biotinylated ODN immobilized on either avidin beads or avidin coated petri dishes. **CpG** ODN conjugated to either FITC or biotin retained full mitogenic properties, indicating no steric hindrance.

DETD Recent data indicate the involvement of the transcription factor

NFκB as a direct or indirect mediator of the **CpG** effect. For example, within 15 minutes of treating B cells or monocytes with **CpG** DNA, the level of NFkB binding activity is increased (FIG. 7). However, it is not increased by DNA that does not contain **CpG** motifs. In addition, it was found that two different inhibitors of NFκB activation, PDTC and gliotoxin, completely block the lymphocyte stimulation by **CpG** DNA as measured by B cell proliferation or monocytic cell cytokine secretion, suggesting that NFκB activation is required for both. . .

DETD . . . various protein kinases, or through the generation of reactive oxygen species. No evidence for protein kinase activation induced immediately after **CpG** DNA treatment of B cells or monocytic cells have been found, and inhibitors of protein kinase A, protein kinase C, and protein tyrosine kinases had no effects on the **CpG** induced activation. However, **CpG** DNA causes a rapid induction of the production of reactive oxygen species in both B cells and monocytic cells, as. . . reactive oxygen species completely block the induction of NFκB and the later induction of cell proliferation and cytokine secretion by **CpG** DNA.

DETD Working backwards, the next question was how **CpG** DNA leads to the generation of reactive oxygen species so quickly. Previous studies by the inventors demonstrated that oligonucleotides and. . . rapidly become acidified inside the cell. To determine whether this acidification step may be important in the mechanism through which **CpG** DNA activates reactive oxygen species, the acidification step was blocked with specific inhibitors of endosome acidification including chloroquine, monensin, and. . . the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the **CpG** oligo also showed an increase in the level of reactive oxygen species such that more than 50% of the cells became positive (Panel D). However, cells treated with an oligonucleotide with the identical sequence except that the **CpG** was switched did not show this significant increase in the level of reactive oxygen species (Panel E).

DETD . . . have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with **CpG** DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and. . . This demonstrates that unlike the PMA plus ionomycin, the generation of reactive oxygen species following treatment of B cells with **CpG** DNA requires that the DNA undergo an acidification step in the endosomes. This is a completely novel mechanism of leukocyte activation. Chloroquine, monensin, and bafilomycin also appear to block the activation of NFκB by **CpG** DNA as well as the subsequent proliferation and induction of cytokine secretion.

DETD Presumably, there is a protein in or near the endosomes that specifically recognizes DNA containing **CpG** motifs and leads to the generation of reactive oxygen species. To detect any protein in the cell cytoplasm that may specifically bind **CpG** DNA, we used electrophoretic mobility shift assays (EMSA) with 5' radioactively labeled oligonucleotides with or without **CpG** motifs. A band was found that appears to represent a protein binding specifically to single stranded oligonucleotides that have **CpG** motifs, but not to oligonucleotides that lack **CpG** motifs or to oligonucleotides in which the **CpG** motif has been methylated. This binding activity is blocked if excess of oligonucleotides that contain the NFκB binding site was. . . suggests that an NFκB or related protein is a component of a protein or protein complex that binds the stimulatory **CpG** oligonucleotides.

DETD . . . points where NFκB was strongly activated. These data therefore do not provide proof that NFκB proteins actually bind to the **CpG** nucleic acids, but rather that the proteins are required in some way for the **CpG** activity. It is possible that a CREB/ATF or related protein may interact in some way with NFκB proteins or other proteins thus explaining the remarkable similarity in the binding motifs for CREB proteins and the optimal **CpG** motif. It remains possible that the oligos bind to a CREB/ATF or related protein, and that this leads to NFκB. . .

DETD Alternatively, it is very possible that the **CpG** nucleic acids may bind to one of the TRAF proteins that bind to the cytoplasmic region of CD40 and mediate. . .

DETD Method for Making **Immunostimulatory** Nucleic Acids

DETD . . . (Uhlmann, E. and Peyman, A. (1990) Chem. Rev. 90:544; Goodchild, J. (1990) Bioconjugate Chem. 1: 165). 2'-O-methyl nucleic acids with **CpG** motifs also cause immune activation, as do ethoxy-modified **CpG** nucleic acids. In fact, no backbone modifications have been found that completely abolish the **CpG** effect, although it is greatly reduced by replacing the C with a 5-methyl C.

DETD Therapeutic Uses of **Immunostimulatory** Nucleic Acid Molecules

DETD Based on their **immunostimulatory** properties, nucleic acid molecules

containing at least one unmethylated **CpG** dinucleotide can be administered to a subject in vivo to treat an "immune system deficiency". Alternatively, nucleic acid molecules containing at least one unmethylated **CpG** dinucleotide can be contacted with lymphocytes (e.g. B cells, monocytic cells or NK cells) obtained from a subject having an. . .

DETD As reported herein, in response to unmethylated **CpG** containing nucleic acid molecules, an increased number of spleen cells secrete IL-6, IL-12, IFNγ, IFNα, IFNβ, IL-1, IL-3, IL-10, TNFα,. . .

DETD **Immunostimulatory** nucleic acid molecules can also be administered to a subject in conjunction with a vaccine to boost a subject's immune system and thereby effect a better response from the vaccine. Preferably the **immunostimulatory** nucleic acid molecule is administered slightly before or at the same time as the vaccine. A conventional adjuvant may optionally. . .

DETD . . . antigen encoded by the vaccine determines the specificity of the immune response. Second, if the backbone of the plasmid contains **CpG** motifs, it functions as an adjuvant for the vaccine. Thus, **CpG** DNA acts as an effective "danger signal" and causes the immune system to respond vigorously to new antigens in the area. This mode of action presumably results primarily from the stimulatory local effects of **CpG** DNA on dendritic cells and other "professional" antigen presenting cells, as well as from the costimulatory effects on B cells.

DETD **Immunostimulatory** oligonucleotides and unmethylated **CpG** containing vaccines, which directly activate lymphocytes and co-stimulate an antigen-specific response, are fundamentally different from conventional adjuvants (e.g. aluminum precipitates),. . .

DETD In addition, an **immunostimulatory** oligonucleotide can be administered prior to, along with or after administration of a chemotherapy or immunotherapy to increase the responsiveness. . . chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF. **CpG** nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Induction of NK activity and. . .

DETD Another use of the described **immunostimulatory** nucleic acid molecules is in desensitization therapy for allergies, which are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated **CpG** nucleic acids are predominantly of a class called "Th1" which is most marked by a cellular immune response and is. . . are mediated by Th2 type immune responses and autoimmune diseases by Th1 immune response. Based on the ability of the **immunostimulatory** nucleic acid molecules to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of an **immunostimulatory** nucleic acid (or a vector containing a nucleic acid) alone or in conjunction with an allergen can be administered to. . .

DETD Nucleic acids containing unmethylated **CpG** motifs may also have significant therapeutic utility in the treatment of asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated. . .

DETD As described in detail in the following Example 12, oligonucleotides containing an unmethylated **CpG** motif (i.e. **TCCATGACGTTCCTGACGTT**; SEQ ID NO. 10), but not a control oligonucleotide (TCCATGAGCTTCCTGAGTCT; SEQ ID NO 11) prevented the development of an inflammatory. . .

DETD For use in therapy, an effective amount of an appropriate **immunostimulatory** nucleic acid molecule alone or formulated as a delivery complex can be administered to a subject by any mode allowing. . .

DETD . . . to realize a desired biologic effect. For example, an effective amount of a nucleic acid containing at least one unmethylated **CpG** for treating an immune system deficiency could be that amount necessary to eliminate a tumor, cancer, or bacterial, viral or. . . such factors as the disease or condition being treated, the particular nucleic acid being administered (e.g. the number of unmethylated **CpG** motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or. . .

DETD . . . Table 1). In some experiments, culture supernatants were assayed for IgM by ELISA, and showed similar increases in response to **CpG**-ODN.

DETD . . . C57BL/6 spleen cells were cultured in two ml RPMI (supplemented as described for Example 1) with or without 40 μM **CpG** or non-**CpG** ODN for forty-eight hours. Cells were washed, and then used as effector cells in a short term ^{51}Cr release. . .

DETD In Vivo Studies with **CpG** Phosphorothioate ODN

DETD B cells were cultured with phosphorothioate ODN with the sequence of control ODN 1a or the **CpG** ODN 1d and 3Db and then either pulsed after 20 hr with ^{3}H uridine or after 44 hr with. . .

DETD . . . for 1 hr. at 37 C in the presence or absence of LPS or the control ODN 1a or the **CpG** ODN 1d and 3Db before addition of anti-IgM

(1 μ/ml). Cells were cultured for a further 20 hr. before a. . .

DETD DBA/2 female mice (2 mos. old) were injected IP with 500 μg **CpG** or control phosphorothioate ODN. At various time points after injection, the mice were bled. Two mice were studied for each. . .

DETD . . . (2U/μg of DNA) at 37° C. for 2 hr in 1×SSC with 5 mM MgCl2. To methylate the cytosine in **CpG** dinucleotides in E. coli DNA, E. coli DNA was treated with **CpG** methylase (M. SssI; 2U/P g of DNA) in NEBuffer 2 supplemented with 160 μM S-adenosyl methionine and incubated overnight at. . .

DETD . . . humidified incubator maintained in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 1.5 mM L-glutamine, 50 μg/ml), **CpG** or non-**CpG** phosphodiester ODN (O-ODN) (20 μM), phosphorothioate ODN (S-ODN) (0.5 μM), or E. coli or calf thymus DNA (50 μg/ml) at. . . IgM production). Concentrations of stimulants were chosen based on preliminary studies with titrations. In some cases, cells were treated with **CpG** O-ODN along with various concentrations (1-10 μg/ml) of neutralizing rat IgG1 antibody against murine IL-6 (hybridoma MP5-20F3) or control rat. . .

DETD . . . injected intravenously (iv) with PBS, calf thymus DNA (200 μg/100 μl PBS/mouse), E. coli DNA (200 μg/100 μl PBS/mouse), or **CpG** or non-**CpG** S-ODN (200 μg/100 μl PBS/mouse). Mice (two/group) were bled by retroorbital puncture and sacrificed by cervical dislocation at various time. . .

DETD Cell Proliferation assay. DBA/2 mice spleen B cells (5×10^4 cells/100 μl/well) were treated with media, **CpG** or non-**CpG** S-ODN (0.5 μM) or O-ODN (20 μM) for 24 hr at 37° C. Cells were pulsed for the last four. . .

DETD . . . a receptor-dependent mechanism. J. Clin. Invest. 93:944) at 250 mV and 960 μF. Cells were stimulated with various concentrations or **CpG** or non-**CpG** ODN after electroporation. Chloramphenicol acetyltransferase (CAT) activity was measured by a solution assay (Seed, B. and J. Y. Sheen (1988). . .

DETD Oligodeoxynucleotide Modifications Determine the Magnitude of B Cell Stimulation by **CpG** Motifs

DETD . . . central linkages are phosphodiester, but the two 5' and five 3' linkages are methylphosphonate modified. The ODN sequences studied (with **CpG** dinucleotides indicated by underlining) include:

DETD These sequences are representative of literally hundreds of **CpG** and non-**CpG** ODN that have been tested in the course of these studies.

DETD . . . (1990) J. Immunol 145:1039; and Ballas, Z. K. and W. Rasmussen (1993) J. Immunol, 150:17), with medium alone or with **CpG** or non-**CpG** ODN at the indicated concentrations, or with E. coli or calf thymus (50 μg/ml) at 37° C. for 24 hr.. . .

DETD . . . mice were then treated with oligonucleotides (30 μg in 200 μl saline by i.p. injection), which either contained an unmethylated **CpG** motif (i.e. **TCCATGACGTTCCTGACGTT**; SEQ ID NO.10) or did not (i.e. control; TCCATGAGCTTCCTGAGTCT; SEQ ID NO.11). Soluble SEA (10 μg in 25 μl of. . .

DETD . . . inflammatory cells are present in the lungs. However, when the mice are initially given a nucleic acid containing an unmethylated **CpG** motif along with the eggs, the inflammatory cells in the lung are not increased by subsequent inhalation of the egg. . .

DETD . . . inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a **CpG** oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes. . .

DETD FIG. 14 shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can actually redirect the cytokine response of the lung to production of II-12, indicating a Th1 type of immune. . .

DETD FIG. 15 shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune. .

DETD **CpG** Oligonucleotides Induce Human PBMC to Secrete Cytokines.

DETD . . . standard centrifugation over ficoll hypaque. Cells (5×10^5 /ml) were cultured in 10% autologous serum in 96 well microtiter plates with **CpG** or control oligodeoxynucleotides (24 μg/ml for phosphodiester oligonucleotides; 6 μg/ml for nuclease resistant phosphorothioate oligonucleotides) for 4 hr in the. . .

. . . deficiency in a subject, comprising the steps of: a) contacting lymphocytes obtained from the subject with an antigen and an **immunostimulatory** nucleic acid, comprising: 5' X_1 CGX_2 3' wherein the **immunostimulatory** nucleic acid includes at least 8 nucleotides and wherein C is unmethylated, wherein X_1 and X_2 are nucleotides and wherein the **immunostimulatory** nucleic acid is an **immunostimulatory** nucleic acid selected from the group consisting of a synthetic **immunostimulatory** nucleic acid and an **immunostimulatory** nucleic acid having a phosphate modified backbone ex vivo, thereby producing activated lymphocytes; and b) readministering the activated

lymphocytes obtained. . .

. . . allergic reaction in response to contact with a particular allergen, comprising administering to the subject an effective amount of an **immunostimulatory** nucleic acid, comprising: 5' X_1 CGX_2 3' wherein the **immunostimulatory** nucleic acid includes at least 8 nucleotides and wherein C is unmethylated and wherein X_1 and X_2 are nucleotides and. . .

. . . in a subject, comprising administering to the subject a vaccine antigen or an antigen encoded in a DNA vaccine and **immunostimulatory** nucleic acid, comprising: 5' X_1 CGX_2 3' wherein the **immunostimulatory** nucleic acid includes at least 8 nucleotides and wherein C is unmethylated and wherein X_1 and X_2 are nucleotides.

5. A method for treating leukemia in a subject, comprising administering to the subject an **immunostimulatory** nucleic acid, comprising: 5' X_1 CGX_2 3' wherein the **immunostimulatory** nucleic acid includes at least 8 nucleotides and wherein C is unmethylated, wherein X_1 and X_2 are nucleotides, prior to. . .

6. A composition comprising: a plasmid including an **immunostimulatory** nucleic acid sequence, comprising: 5'X_1 X_2 CGX_3 X_4 3' wherein C is unmethylated, wherein X_1, X_2, X_3 and X_4 are. . .

14. A composition comprising: an isolated **immunostimulatory** nucleic acid of 8 to 100 nucleotides in length, comprising: 5' X_1 CGX_2 3' wherein C is unmethylated, wherein X_1. . .

15. The composition of claim 14, wherein the isolated **immunostimulatory** nucleic acid includes a phosphate backbone modification which is a phosphorothioate or phosphorodithioate modification.

18. A composition comprising: an **immunostimulatory** nucleic acid comprising: 5' X_1 CGX_2 3' wherein the **immunostimulatory** nucleic acid includes at least 8 nucleotides and wherein C is unmethylated, wherein X_1 and X_2 are nucleotides, and an. . .

19. The composition of claim 18, wherein the synthetic **immunostimulatory** nucleic acid includes a phosphate backbone modification which is a phosphorothioate or phosphorodithioate modification.

22. A composition comprising: an **immunostimulatory** nucleic acid of 8 to 40 nucleotides in length, comprising: 5' X_1 CGX_2 3' wherein C is unmethylated, wherein X_1. . .

23. The composition of claim 22, wherein the **immunostimulatory** nucleic acid includes a phosphate backbone modification which is a phosphorothioate or phosphorodithioate modification.

. . . in a subject comprising: administering a vaccine to a subject, wherein the vaccine includes an antigen in combination with an **immunostimulatory** nucleic acid of claims 6, 14, 18 or 22 in an amount effective to induce an immune response.

28. The method of claim 27, wherein leukocytes of the subject are isolated and contacted with the antigen and **immunostimulatory** nucleic acid to produce activated leukocytes and wherein the activated leukocytes are readministered to the subject.

30. The composition of claim 18, wherein the **immunostimulatory** nucleic acid molecule is 8 to 100 nucleotides in length.

31. A composition comprising: an **immunostimulatory** nucleic acid, comprising: 5' X_1 X_2 CGX_3 X_4 3' wherein the **immunostimulatory** nucleic acid includes at least 8 nucleotides, wherein C is unmethylated, wherein X_1, X_2, X_3 and X_4 are nucleotides, and. . .

32. The composition of claim 31, wherein the **immunostimulatory** nucleic acid molecule is 8 to 100 nucleotides in length.

. . . 31, wherein X_1 X_2 are dinucleotides selected from the group consisting of: GpT, GpG, GpA ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG.

. . . composition of claim 31, wherein X_3 X_4 are dinucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

35. The composition of claim 31, wherein the **immunostimulatory** nucleic acid is associated with a cationinc lipid.

L15 ANSWER 11 OF 12 USPATFULL on STN
2000:28125 Nucleic acids encoding myocardial peptides.
Bachmaier, Kurt, Toronto, Canada
Hessel, Andrew John, Toronto, Canada
Neu, Nickolaus, Innsbruck, Austria
Penninger, Josef Martin, Toronto, Canada
Amgen Canada Inc., Mississauga, Canada (non-U.S. corporation)
US 6034230 20000307
APPLICATION: US 1999-303862 19990503 (9)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed:
1. An isolated nucleic acid molecule selected from the group consisting of: SEQ ID NO:17, SEQ ID NO: 18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:24.

2. A vector comprising the isolated nucleic acid molecule of claim 1.

3. A host cell comprising the vector of claim 2.

4. An isolated nucleic acid molecule consisting of SEQ ID NO:25 or SEQ ID NO: 26.

5. A vector comprising the isolated nucleic acid molecule of claim 4.

6. A host cell comprising the vector of claim 5.

AI US 1999-303862 19990503 (9)
SUMM The term "**CpG** oligodeoxynucleotide" refers to an oligodeoxynucleotide containing the internal motif "GACGTT". Preferably, the **CpG** oligodeoxynucleotide will be about 20 nucleotides in length, but may range from about 14 to 30 or more nucleotides in. . .
SUMM . . . a gene encoding inflammatory cardiomyopathy peptide or fragment thereof, or therapeutic cardiomyopathy peptide or fragment thereof, or to prepare a **CpG** oligodeoxynucleotide of the present invention, is to employ chemical synthesis using methods well known to the skilled artisan such as. . .
SUMM Certain bacterial DNA molecules purportedly can have **immunostimulatory** effects in vivo and in vitro (Davis et al., J. Immunol., 160:870-876 [1998]). However, prior to the present invention, it was not known that certain oligodeoxynucleotides having a **CpG** motif (GACGTT) could be useful as adjuvants for vaccines.
SUMM **TCCATGACGTTCCTGACGTT** (SEQ ID NO:12)
DETD . . . kDa cysteine rich outer membrane protein from Chlamydia trachomatis (de la Maza et al., Infect. Immun., 59:1196-1201 [1991]) containing a **CpG** motif and referred to as a "**CpG** oligo" (SEQ ID NO:13), and its counterpart not containing the **CpG** motif, the "non-**CpG** oligo" (SEQ ID NO:14), were synthesized using standard phosphoramidite chemistry, and were phosphorothioate modified (Stein et al., supra; Caruthers et. . .
DETD . . . 50 micrograms of M7A-alpha peptide (SEQ ID NO:2) and about 10 nmol of the oligodeoxynucleotide of either SEQ ID NO:13 (**CpG** oligodeoxynucleotide) or SEQ ID NO:14 (non-**CpG** oligodeoxynucleotide), together with Freund's incomplete adjuvant. Negative control mice received about 10 nmol of the oligodeoxynucleotide of SEQ ID NO:13. .

DETD TABLE 2

Adjuvant	Peptide	Prevalence	Severity
CFA	M7A-alpha	5/5	3.8 ± 0.4
CpG	M7A-alpha	5/5	1.2 ± 0.4
non-**CpG**	M7A-alpha	1/5	1.0 ± 0.0
CpG	None	0/5	--

DETD Surprisingly, the **CpG** oligonucleotide plus M7A-alpha peptide induced inflammatory heart disease in the absence of Freund's complete adjuvant, indicating that this oligonucleotide, which contains the **CpG** motif, can serve as a potent immunostimulator. The oligonucleotide containing the non-**CpG** motif was hardly effective as an adjuvant. Other **CpG** oligodeoxynucleotides tested and found to be **immunostimulatory** include the oligos set forth in SEQ ID Nos:10-12 (see above).
DETD . . . rich outer membra - #ne protein from Chlamydia trachomatis containing a C - #pG motif and referred to as a **CpG** oligo.
- - <400> SEQUENCE: 13

- - gtactgacgt ttactcttgg - # - #
- # 20
- - -. . . encoding a 60 kDa cysteine rich - #outer membrane protein from Chlamydia trachomatis which does not - # contain the **CpG** motif and referred to as a - # non-**CpG** oligo.
- - <400> SEQUENCE: 14
- - gtactgagct ttactcttgg - # - #
- # 20
- - -. . .

L15 ANSWER 12 OF 12 USPATFULL on STN
1999:121537 Peptides capable of modulating inflammatory heart disease.
Bachmaier, Kurt, Toronto, Canada
Hessel, Andrew John, Toronto, Canada
Neu, Nickolaus, Innsbruck, Austria
Penninger, Josef Martin, Toronto, Canada
Amgen Canada Inc., Mississauga, Canada (non-U.S. corporation)
US 5962636 19991005
APPLICATION: US 1998-133774 19980812 (9)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed:
1. A peptide selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:16.

2. The peptide of claim 1 wherein the amino-terminal amino acid is acylated.

3. The peptide of claim 2 wherein an acetyl group is used for acylation.

4. A peptide selected from the group consisting of: SEQ ID NO:3 and SEQ ID NO:15.

5. The peptide of claim 4 wherein the amino-terminal amino acid is acylated.

6. The peptide of claim 5 wherein an acetyl group is used for acylation.

7. A vaccine to decrease inflammatory cardiomyopathy comprising a peptide, an adjuvant, and an excipient, wherein the peptide consists of any of SEQ ID NOS; 2, 3, 4, 5, 6, 7, 8, 9, 15, or 16.

AI US 1998-133774 19980812 (9)
SUMM The term "**CpG** oligodeoxynucleotide" refers to an oligodeoxynucleotide containing the internal motif "GACGTT". Preferably, the **CpG** oligodeoxynucleotide will be about 20 nucleotides in length, but may range from about 14 to 30 or more nucleotides in. . .
SUMM . . . a gene encoding inflammatory cardiomyopathy peptide or fragment thereof, or therapeutic cardiomyopathy peptide or fragment thereof, or to prepare a **CpG** oligodeoxynucleotide of the present invention, is to employ chemical synthesis using methods well known to the skilled artisan such as. . .
SUMM Certain bacterial DNA molecules purportedly can have **immunostimulatory** effects in vivo and in vitro (Davis et al., J. Immunol. , 160: 870 -876 [1998]). However, prior to the present invention, it was not known that certain oligodeoxynucleotides having a **CpG** motif (GACGTT) could be useful as adjuvants for vaccines.
SUMM **TCCATGACGTTCCTGACGTT** (SEQ ID NO:12)
DETD . . : cysteine rich outer membrane protein from Chlamydia trachomatis (de la Maza et al., Infect. Immun., 59: 1196-1201 [1991]) containing a **CpG** motif and referred to as a "**CpG** oligo" (SEQ ID NO:13), and its counterpart not containing the **CpG** motif, the "non-**CpG** oligo" (SEQ ID NO:14), were synthesized using standard phosphoramidite chemistry, and were phosphorothioate modified (Stein et al., supra; Caruthers et. . .
DETD . . . 50 micrograms of M7A-alpha peptide (SEQ ID NO:2) and about 10 nmol of the oligodeoxynucleotide of either SEQ ID NO:13 (**CpG** oligodeoxynucleotide) or SEQ ID NO:14 (non-**CpG** oligodeoxynucleotide), together with Freund's incomplete adjuvant. Negative control mice received about 10 nmol of the oligodeoxynucleotide of SEQ ID NO:13. .
DETD TABLE 2

Adjuvant	Peptide	Prevalence	Severity
CFA	M7A-alpha	5/5	3.8 ± 0.4
CPG	M7A-alpha	5/5	1.2 ± 0.4

non-**CpG** M7A-alpha 1/5 1.0 ± 0.0
CpG None 0/5 --

DETD Surprisingly, the **CpG** oligonucleotide plus M7A-alpha peptide induced inflammatory heart disease in the absence of Freund's complete adjuvant, indicating that this oligonucleotide, which contains the **CpG** motif, can serve as a potent immunostimulator. The oligonucleotide containing the non-**CpG** motif was hardly effective as an adjuvant. Other **CpG** oligodeoxynucleotides tested and found to be **immunostimulatory** include the oligos set forth in SEQ ID Nos:10-12 (see above).
DETD . . . - # the DNA
#outer membrane protein fromeine rich
Chlamydia trachomatis containing a C - #pG motif and referred to as **CpG** oligo.
- <400> SEQUENCE: 13
20 ttgg
- <210> SEQ ID NO 14
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Chlamydia trachomatis
<220> FEATURE:
#from the DNANFORMATION: An oligonucleotide derived
#outer membrane protein fromeine rich
Chlamydia trachomatis which does not - # contain the **CpG** motif and referred to as a non-**CpG** oligo.
- <400> SEQUENCE: 14
20 ttgg
- <210> SEQ ID NO 15
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Human
- <400> SEQUENCE: 15
-. . .

=> d his

(FILE 'HOME' ENTERED AT 00:18:42 ON 13 NOV 2006)

FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006
E GARCON NATALIE/IN
L1 25 S E4-E7
L2 8 S L1 AND (CPG)
L3 17 S L1 NOT L2
E FRIEDE MARTIN/IN
L4 17 S E3
L5 12 S L4 NOT L1
L6 12 S (WD1001 OR WD1002 OR WD1003 OR WD1004 OR WD1005 OR WD1006 OR
L7 11 S L6 NOT L1
L8 11 S L7 NOT L4
L9 1 S L8 AND (CPG)
L10 154 S (TCCATGACGTTCCTGACGTT)
L11 154 S L10 NOT L1
L12 154 S L11 NOT L4
L13 154 S L12 AND (CPG)
L14 138 S L13 AND (IMMUNOSTIMULATORY)
L15 12 S L14 AND AY<2000

=> s (TCTCCCAGCGTGCGCCAT)
L16 110 (TCTCCCAGCGTGCGCCAT)

=> s l16 and l15
L17 8 L16 AND L15

=> d l17,cbib,1-8

L17 ANSWER 1 OF 8 USPATFULL on STN
2006:127412 Compositions of **CPG** and saponin adjuvants and uses thereof.
Kensil, Charlotte A., Milford, MA, UNITED STATES
Antigenics Inc., Lexington, MA, UNITED STATES (U.S. corporation)
US 7049302 B1 20060523
APPLICATION: US 1999-369941 19990806 (9)
PRIORITY: US 1999-128608P 19990408 (60)
US 1998-95913P 19980810 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 2 OF 8 USPATFULL on STN
2003:309071 Method of treating cancer using **immunostimulatory** oligonucleotides

Krieg, Arthur M., Iowa City, IA, United States
Weiher, George, Iowa City, IA, United States
University of Iowa Research Foundation, Iowa City, IA, United States (U.S. corporation)
US 6653292 B1 20031125
APPLICATION: US 1999-337619 19990621 (9)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 3 OF 8 USPATFULL on STN
2002:194879 **Immunostimulatory** nucleic acid molecules for activating dendritic cells.
Krieg, Arthur M., Iowa City, IA, United States
Hartmann, Gunther, Munchen, GERMANY, FEDERAL REPUBLIC OF
University of Iowa Research Foundation, Iowa City, IA, United States (U.S. corporation)
US 6429199 B1 20020806
APPLICATION: US 1998-191170 19981113 (9)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 4 OF 8 USPATFULL on STN
2002:143951 Use of nucleic acids containing unmethylated **CpG** dinucleotide as an adjuvant.
Davis, Heather L., Ottawa, CANADA
Schorr, Joachim, Hilden, GERMANY, FEDERAL REPUBLIC OF
Krieg, Arthur M., Iowa City, IA, United States
University of Iowa Research Foundation, Iowa City, IA, United States (U.S. corporation)Coley Pharmaceutical GmbH, Langenfield, GERMANY, FEDERAL REPUBLIC OF (non-U.S. corporation)Ottawa Health Research Institute, Ottawa, CANADA (non-U.S. corporation)
US 6406705 B1 20020618
APPLICATION: US 1999-325193 19990603 (9)
PRIORITY: US 1997-40376P 19970310 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 5 OF 8 USPATFULL on STN
2001:79141 **Immunostimulatory** nucleic acid molecules.
Krieg, Arthur M., Iowa City, IA, United States
Kline, Joel N., Iowa City, IA, United States
University of Iowa Research Foundation, Iowa City, IA, United States (U.S. corporation)Coley Pharmaceutical Group, Inc., Wellesley, MA, United States (U.S. corporation)The United States of America as represented by the Department of Health and Human Services, Washington, DC, United States (U.S. government)
US 6239116 B1 20010529
APPLICATION: US 1997-960774 19971030 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 6 OF 8 USPATFULL on STN
2001:55947 Methods and products for stimulating the immune system using immunotherapeutic oligonucleotides and cytokines.
Krieg, Arthur M., Iowa City, IA, United States
Weiner, George, Iowa City, IA, United States
University of Iowa Research Foundation, Iowa City, IA, United States (U.S. corporation)
US 6218371 B1 20010417
APPLICATION: US 1999-286098 19990402 (9)
PRIORITY: US 1998-80729P 19980403 (60)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 7 OF 8 USPATFULL on STN
2001:52030 Use of nucleic acids containing unmethylated CPC dinucleotide in the treatment of LPS-associated disorders.
Krieg, Arthur M., Iowa City, IA, United States
Schwartz, David A., Iowa City, IA, United States
University of Iowa Research Foundation, Iowa City, IA, United States (U.S. corporation)
US 6214806 B1 20010410
APPLICATION: US 1998-30701 19980225 (9)
PRIORITY: US 1997-39405P 19970228 (60)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 8 OF 8 USPATFULL on STN

2001:44204 **Immunostimulatory** nucleic acid molecules.
Krieg, Arthur M., Iowa City, IA, United States
Kline, Joel, Iowa City, IA, United States
Klinman, Dennis, Potomac, MD, United States
Steinberg, Alfred D., Potomac, MD, United States
University of Iowa Research Foundation, Iowa City, IA, United States (U.S. corporation)Coley Pharmaceutical Group, Inc., Wellesley, MA, United States (U.S. corporation)The United States of America as represented by the Department of Health and Human Services, Washington, DC, United States (U.S. government)
US 6207646 B1 20010327
APPLICATION: US 1996-738652 19961030 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

=> d l17,cbib,clm,kwic,1-5

L17 ANSWER 1 OF 8 USPATFULL on STN
2006:127412 Compositions of **CPG** and saponin adjuvants and uses thereof.
Kensil, Charlotte A., Milford, MA, UNITED STATES
Antigenics Inc., Lexington, MA, UNITED STATES (U.S. corporation)
US 7049302 B1 20060523
APPLICATION: US 1999-369941 19990806 (9)
PRIORITY: US 1999-128608P 19990408 (60)
US 1998-95913P 19980810 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:

1. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide is not a part of a DNA vaccine vector, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

2. The immune adjuvant composition as claimed in claim 1, wherein the saponin comprises a substantially pure saponin.

3. The immune adjuvant composition as claimed in claim 2, wherein the substantially pure saponin is QS-7, QS-17, QS-18, or QS-21.

4. The immune adjuvant composition as claimed in claim 3, wherein the substantially pure saponin is QS-21.

5. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises more than one unmethylated **CpG** dinucleotide.

6. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester.

7. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

8. The immune adjuvant composition as claimed in claim 1, wherein the **immunostimulatory** oligonucleotide comprises a **CpG** motif having the formula $5'X_1CGX_2 3'$, wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

9. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1).

10. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the saponin is substantially pure, and the saponin is QS-7, QS-17 or QS-18, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

11. A method for inducing an immune response in an individual to an

antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 10 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

12. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

13. The immune adjuvant composition as claimed in claim 12, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

14. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 12 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

15. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 13 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

16. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTFCGCCAT (SEQ ID NO:1), and, wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

17. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 16 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

18. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide comprises **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2), and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

19. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 18 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

20. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide is 4-40 bases in length, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

21. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 20 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

22. An immune adjuvant composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin (i) is derived from Quillaja saponaria and (ii) is a chemically modified saponin; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

23. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 22 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

24. The composition of claim 1, wherein the saponin is a chemically modified saponin.

25. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

26. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 1 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

27. The method as claimed in any of claims 14, 15, 17, 19, 21, 23, or 26, wherein the saponin comprises is a substantially pure saponin.

28. The method as claimed in claim 27, wherein the substantially pure saponin is QS-7, QS-17, QS-18, or QS-21.

29. The method as claimed in claim 28, wherein the substantially pure saponin is QS-21.

30. The method as claimed in any of claims 11, 14, 15, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises more than one unmethylated **CpG** dinucleotide.

31. The method as claimed in any of claims 11, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phosphorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester.

32. The method as claimed in any of claims 11, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

33. The method as claimed in any of claims 11, 14, 15, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises a **CpG** motif having the formula 5'$X_1CGX_2$3', wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

34. The method as claimed in any of claims 11, 14, 15, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

35. The method as claimed in any of claims 11, 14, 15, 21, 19, 21, 23, or 26, wherein the individual is an animal.

36. The method as claimed in claim 35, wherein the animal is a mammal.

37. The method as claimed in any of claims 11, 14, 15, 21, 19, 21, 23, or 26, wherein the individual is a human.

38. A vaccine composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide; and (c) a nucleic acid molecule comprising a nucleotide sequence encoding an antigen, wherein the nucleotide sequence is operatively linked to a promoter, wherein the **immunostimulatory** oligonucleotide is not a part of the nucleic acid molecule comprising the nucleotide sequence encoding the antigen.

39. The vaccine composition as claimed in claim 38, wherein the saponin is a substantially pure saponin.

40. The vaccine composition as claimed in claim 39, wherein the substantially pure saponin is QS-7, QS-17, QS-18, or QS-21.

41. The vaccine composition as claimed in claim 40, wherein the substantially pure saponin is QS-21.

42. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises more than one unmethylated **CpG** dinucleotide.

43. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester.

44. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

45. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises a **CpG** motif having the formula $5'X_{1CGX23}'$, wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

46. The vaccine composition as claimed in claim 38 or 41, wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

47. The method of any of claims 11, 17, 19, 23, or 26, wherein the nucleic acid molecule encoding the antigen is administered to the individual concurrently with the immune adjuvant composition.

48. The method of any of claims 14, 15, or 21, wherein the nucleic acid molecule encoding the antigen is administered to the individual concurrently with the immune adjuvant composition.

49. The method as claimed in any of claims 14, 15, 21, 23, and 26, wherein the saponin is substantially pure, wherein the saponin is QS-21, and wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

50. The immune adjuvant composition as claimed in claim 12 or 20, wherein the saponin is chemically modified.

51. The immune adjuvant composition as claimed in claim 12, 20 or 22, wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

52. The immune adjuvant composition as claimed in claim 12 or 22, wherein the saponin is substantially pure.

53. The immune adjuvant composition as claimed in claim 52, wherein the saponin is QS-21.

54. The immune adjuvant composition as claimed in claim 53, wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

55. The immune adjuvant composition as claimed in claim 20, wherein the saponin is substantially pure.

56. The immune adjuvant composition as claimed in claim 55, wherein the saponin is QS-21.

57. The immune adjuvant composition as claimed in claim 20 or 56, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester.

58. The immune adjuvant composition as claimed in claim 56, wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ

ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

59. The immune adjuvant composition as claimed in claim 16 or 18, wherein the saponin is substantially pure.

60. The immune adjuvant composition as claimed in claim 59, wherein the saponin is QS-21.

TI Compositions of **CPG** and saponin adjuvants and uses thereof
AI US 1999-369941 19990806 (9)
AB Vaccine compositions of **immunostimulatory** oligonucleotides and saponin adjuvants and antigens and the use thereof for stimulating immunity, enhancing cell-mediated immunity, and enhancing antibody production are disclosed. Also described are immune adjuvant compositions comprising **immunostimulatory** oligonucleotides and saponin adjuvants, as well as methods for increasing an immune response using the same.
SUMM Recently, oligonucleotides containing the unmethylated cytosine-guanine ("**CpG**") dinucleotide in a particular sequence context or motif have been shown to be potent stimulators of several types of immune cells in vitro. (Weiner, et al., Proc. Natl. Acad. Sci. 94:10833 (1997).) An **immunostimulatory** oligonucleotide comprising an unmethylated **CpG** motif is an dinucleotide within the oligonucleotide that consistently triggers an **immunostimulatory** response and release of cytokines. **CpG** motifs can stimulate monocytes, macrophages, and dendritic cells that can produce several cytokines, including the T helper 1 ("Th 1"). . . et al., J. Exp. Med. 186:1623 (1997).) Klinman, et al., have shown that a DNA motif consisting of an unmethylated **CpG** dinucleotide flanked by two 5' purines (GpA or ApA) and two 3' pyrimidines (TpC or TpT) optimally stimulated B cells. . . et al., the contents of which are incorporated herein by reference, discovered that nucleic acids containing at least one unmethylated **CpG** dinucleotide may affect the immune response of a subject (Davis, et al., WO 98/40100, PCT/US98/04703).
SUMM . . . adjuvants may be potentially incorporated in future human vaccines. Surprisingly, a combination of an oligonucleotide comprising at least one unmethylated **CpG** dinucleotide and a saponin adjuvant was found to be a powerful stimulator of cell-mediated immunity compared to either adjuvant alone. Antibody titers (antigen-specific) in response to vaccination were significantly higher for vaccines comprising a **CpG**-containing oligonucleotide/saponin adjuvant combination compared to either saponin or **CpG** alone and represented a positive synergistic adjuvant effect. Together, these results establish that an immune adjuvant composition comprising an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide and a saponin adjuvant is a candidate adjuvant composition for vaccines to induce immunity. Accordingly, the present invention provides novel vaccine compositions which comprise an **immunostimulatory** oligonucleotide, a saponin adjuvant, and an antigen. Methods for increasing the immune response to an antigen by administrating the inventive. . .
DRWD FIG. 1 depicts a graph showing the enhancement of a cell-mediated immune response by QS-21 and **CpG** oligonucleotide/QS-21 combination, as evidenced by the CTL induction.
DRWD FIG. 2 provides a graph showing the enhancement of a cell-mediated immune response by QS-21 and **CpG** oligonucleotide/QS-21 combination, as evidenced by the CTL induction.
DRWD . . . graph of IgG1 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and for combinations of QS-21 and **CpG** oligonucleotide in mouse sera collected 21 days after a first immunization given on day 0.
DRWD . . . bar graph of IgG2a titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and **CpG** oligonucleotide in mouse sera collected 21 days after a first immunization given on day 0.
DRWD . . . bar graph of IgG3 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and **CpG** oligonucleotide in mouse sera collected 21 days after a first immunization given on day 0.
DRWD . . . bar graph of IgG1 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and **CpG** oligonucleotide in mouse sera collected 14 days after a second immunization given 28 days after the first immunization.
DRWD . . . bar graph of IgG2a titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and **CpG** oligonucleotide in mouse sera collected 14 days after a second immunization given 28 days after the first immunization.
DRWD . . . bar graph of IgG3 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and **CpG** oligonucleotide in mouse sera collected 14 days after a second immunization given 28 days after the first immunization.

DETD The present invention may also employ **immunostimulatory** saponins isolated from other plant species. For example, a saponin from Dolichos lablab has been shown to be useful as. . .

DETD The term "**immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide" means an oligonucleotide that has been shown to activate the immune system. The **immunostimulatory** oligonucleotide may, preferably, comprise at least one unmethylated **CpG** dinucleotide. A "**CpG** motif" is a stretch of DNA comprising one or more **CpG** dinucleotides within a specified sequence. The oligonucleotide comprising the **CpG** motif may be as short as 4-40 base pairs in length. The **immunostimulatory** oligonucleotide containing the **CpG** motif may be a monomer or part of a multimer. Alternatively, the **CpG** motif may be a part of the sequence of a vector that also presents a DNA vaccine. It may be. . . double-stranded. It may be prepared synthetically or produced in large scale in plasmids. One embodiment of the invention covers the **immunostimulatory** oligonucleotide which contains a **CpG** motif having the formula 5'$X_1CGX_2$3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine or adenine. In a preferred embodiment, the **CpG** motif comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1; also known as "1758") or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2; also known as "1826").

DETD DNA containing unmethylated **CpG** dinucleotide motifs in the context of certain flanking sequences has been found to be a potent stimulator of several types. . . (1996); Cowdrey, et al., J. Immunol. 156:4570 (1996); Krieg, et al., Nature 374:546 (1995).) Depending on the flanking sequences, certain **CpG** motifs may be more **immunostimulatory** for B cell or T cell responses, and preferentially stimulate certain species. When a humoral response is desired, preferred **immunostimulatory** oligonucleotides comprising an unmethylated **CpG** motif will be those that preferentially stimulate a B cell response. When cell-mediated immunity is desired, preferred **immunostimulatory** oligonucleotides comprising at least one unmethylated **CpG** dinucleotide will be those that stimulate secretion of cytokines known to facilitate a CD8+ T cell response.

DETD The **immunostimulatory** oligonucleotides of the invention may be chemically modified in a number of ways in order to stabilize the oligonucleotide against. . . of the oligonucleotide have been replaced with any number of non-traditional bases or chemical groups, such as phosphorothioate-modified nucleotides. The **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide may preferably be modified with at least one such phosphorothioate-modified nucleotide. Oligonucleotides with phosphorothioate-modified linkages may be prepared using. . .

DETD In a first aspect of the invention, an immune adjuvant composition comprising a saponin adjuvant and an **immunostimulatory** oligonucleotide may be administered. More preferably, such immune adjuvant composition may increase the immune response to an antigen in an. . . saponin adjuvant is QS-21. Alternatively, the immune adjuvant composition may comprise more than one substantially pure saponin adjuvant with the **immunostimulatory** oligonucleotide. In a further preferred embodiment, the saponin adjuvant may cover a chemically modified saponin adjuvant or a fraction thereof. . . at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin retains adjuvant activity. The **immunostimulatory** oligonucleotide, preferably, compries at least one unmethylated **CpG** dinucleotide. The **CpG** dinucleotide is preferably a monomer or multimer. Another preferred embodiment of the **CpG** motif is as a part of the sequence of a vector that also presents a DNA vaccine. Yet another embodiment of the immune adjuvant composition is directed to the **immunostimulatory** oligonucleotide, wherein the **immunostimulatory** oligonucleotide is modified. The particular modification may comprise at least one phosphorothioate-modified nucleotide. Further, the **immunostimulatory** oligonucleotide having at least one unmethylated **CpG** dinucleotide may comprise a **CpG** motif having the formula 5'$X_1CGX_2$3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. The **CpG** motif may preferentially be **TCTCCCAGCGTGCGCCAT** [SEQ ID NO.:1] or **TCCATGACGTTCCTGACGTT** [SEQ ID NO.:2]

DETD . . . the antigen is administered comprising administering an effective amount of an immune adjuvant composition comprising a saponin adjuvant and an **immunostimulatory** oligonucleotide further. Preferably, the saponin adjuvant is a saponin from Quillaja saponaria Molina. More preferably, the saponin adjuvant is a. . . saponaria Molina. The method may also embody an immune adjuvant composition comprising more than one substantially pure saponin adjuvant and **immunostimulatory** oligonucleotide. The substantially pure saponin adjuvant is preferably QS-7, QS-17, QS-18, or QS-21. Most preferably, the substantially pure saponin adjuvant. . . QS-21-V1, and QS-21-V2, and wherein the

chemically modified saponin retains adjuvant activity. In a preferred embodiment of the method, the **immunostimulatory** oligonucleotide comprises at least one unmethylated **CpG** dinucleotide. The **CpG** motif is preferably a monomer or a multimer. Another preferred embodiment of the method includes the **CpG** motif as a part of the sequence of a vector that presents a DNA vaccine. Yet another embodiment is directed to the method wherein the **immunostimulatory** oligonucleotide comprises at least one unmethylated **CpG** dinucleotide, and wherein furthermore, the **immunostimulatory** oligonucleotide may be chemically modified to stabilize the oligonucleotide against endogenous endonucleases. The modification may comprise at least one phosphorothioate-modified nucleotide. Further, the method may be directed, in part, to the **immunostimulatory** oligonucleotide having at least one unmethylated **CpG** dinucleotide comprising a **CpG** motif having the formula 5'$X_1CGX_2$3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. In another preferred method, the unmethylated **CpG** motif is TCTCCCATCGTGCGCCAT [SEQ ID NO.:1] or **TCCATGACGTTCCTGACGTT** [SEQ ID NO.:2]

DETD . . . response. A vaccine composition, according to the invention, would produce immunity against disease in individuals. The combination of saponin and **immunostimulatory** oligonucleotide of the present invention may be administered to an individual to enhance the immune response to any antigen. Preferably,. . .

DETD . . . the invention may enhance antibody production to an antigen in a positive synergistic manner. The synergistic adjuvant effect of the **immunostimulatory** oligonucleotide and the saponin adjuvant described herein may be shown in a number of ways. For example, a synergistic adjuvant. . .

DETD Accordingly, in a third aspect, the invention also encompasses a vaccine composition comprising a saponin adjuvant, an **immunostimulatory** oligonucleotide, and an antigen. The saponin adjuvant may be partially pure or substantially pure saponin from Quillaja saponaria Molina. The vaccine compositions may also comprise more than one partially pure or substantially pure saponin adjuvant, an **immunostimulatory** oligonucleotide further comprising at least one unmethylated **CpG** motif, and an antigen. Preferably, the partially pure saponin adjuvant comprises QS-7, QS-17, QS-18, and/or QS-21 and may comprise other. . . retains adjuvant activity. Most preferably, the partially pure or substantially pure saponin adjuvant in the vaccine composition is QS-21. The **immunostimulatory** oligonucleotide may preferably comprise at least one unmethylated **CpG** dinucleotide. The **CpG** motif may preferably be a monomer or a multimer. Another preferred embodiment of the **CpG** motif is as a part of the sequence of a vector that also presents a DNA vaccine. Yet another embodiment of the vaccine composition described herein is directed to the **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide comprises a chemical modification. More particularly, the **immunostimulatory** oligonucleotide may be modified with at least one phosphorothioate-modified nucleotide. Further, the **immunostimulatory** oligonucleotide having at least one unmethylated **CpG** dinucleotide of the vaccine composition comprises a **CpG** motif having the formula 5'$X_1CGX_2$3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. The unmethylated **CpG** motif according to this aspect of the invention may preferentially comprise **TCTCCCAGCGTGCGCCAT** [SEQ ID NO.:1]or **TCCATGACGTTCCTGACGTT** [SEQ ID NO.:2]

DETD . . . an effective amount of a vaccine composition comprising an antigen, a partially pure or substantially pure saponin adjuvant, and an **immunostimulatory** oligonucleotide. The method also embodies a vaccine composition comprising more than one partially pure or substantially pure saponin adjuvant, an **immunostimulatory** oligonucleotide, and an antigen. Preferably, the partially pure saponin adjuvant comprises QS-7, QS-17, QS-18, and/or QS-21 and may comprise other. . . QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin retains adjuvant activity. Preferably, the method comprises administering an **immunostimulatory** oligonucleotide which further comprises at least one unmethylated **CpG** dinucleotide. The **CpG** dinucleotide therein is a monomer or a multimer. Another preferred embodiment of the method includes the **CpG** motif as a part of the sequence of a vector that also presents a DNA vaccine. Yet another embodiment of the method disclosed herein is directed to the **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide may be chemically modified to increase its stability to endogenous endonucleases. Such a modification may comprise at least one phosphorothioate-modified nucleotide. Further, the **immunostimulatory** oligonucleotide having at least one unmethylated **CpG** dinucleotide may comprise a **CpG** motif having the formula 5'$X_1CGX_2$3', wherein

at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. In another preferred embodiment, the unmethylated **CpG** motif is **TCTCCCAGCGTGCGCCAT** [SEQ ID NO.:1] or **TCCATGACGTTCCTGACGTT** [SEQ ID NO.:2]

DETD A well-established animal model was used to assess whether formulations of **CpG** oligonucleotide and QS-21 together could function as an immune adjuvant. In brief, experiments were set up to compare QS-21 to the recently reported adjuvant **CpG** motif. A **CpG** sequence (e.g., 1758), reported to serve as an adjuvant for a B-cell lymphoma idiotype-KLH vaccine in mice, was selected. One experiment evaluated whether the **CpG** motif, alone or in combination with QS-21, can serve as an adjuvant for a subunit vaccine, e.g., OVA, in mice in inducing CTL responses. This work included a dose range experiment with **CpG** to determine the optimum dose.

DETD In addition to comparing **CpG** and QS-21 as adjuvants, a second experiment combining **CpG** oligonucleotide with suboptimal doses of QS-21 (e.g., 1.25 μg) was conducted to assess whether **CpG** oligonucleotide can affect the adjuvant effect of QS-21.

DETD Also, an experiment was performed to determine whether the **CpG** and QS-21 combination could enhance antibody production, specifically the isotype profile of a antigen-specific antibody response.

DETD Finally, a series of experiments were performed to determine whether a combination of **CpG** oligonucleotide and saponin would enhance antibody production in a positive synergistic manner. This work used vaccine formulations of pneumococcal Type 14 polysaccharide and QS-21 and **CpG** oligonucleotide and evaluated specific antibody titers harvested from mice on days 21 and 42 after immunization on days 0 and 28. Another **CPG** sequence (e.g., 1826), reported to serve as an adjuvant for hen egg lysozyme in mice, was selected.

DETD . . . experiments were done using materials from the following suppliers: OVA, Grade VI (Sigma); pneumococcal Type 14 polysaccharide (ATCC); QS-21 (Aquila); **CpG** oligonucleotides included the phosphorothiate-modified sequence 1758 **TCTCCCAGCGTGCGCCAT** [SEQ ID NO.:1] and phosphorothiate-modified sequence 1826 **TCCATGACGTTCCTGACGTT** [SEQ ID NO.:2] (Life Technologies (Gibco)).

DETD CTL Induced by OS-21 and **CpG**/OS-21

DETD . . . 25 μg OVA antigen plus the indicated doses of adjuvant in a total volume of 0.2 ml phosphate-buffered saline. The **CpG** motif used in this experiment was a phosphorothioate-modified oligonucleotide 1758 with a sequence of **TCTCCCAGCGTGCGCCAT** [SEQ ID NO.:1] (Weiner, et al., Proc. Natl. Acad. Sci. 94:10833 (1997).) Splenocytes were removed at day 42 for use. . .

DETD The results, as shown in FIG. 1, indicate that no lysis was observed in the absence of adjuvant, with any **CpG** dose, or with 1.25 μg of QS-21 (suboptimal dose). However, the suboptimal dose of QS-21, in combination with **CpG**, induced significant CTL. The results show a substantial adjuvant effect at doses that are normally not expected to produce such an adjuvant effect. This positive synergistic effect was most notable at the higher dose of **CpG** (50 μg). The adjuvant effect was comparable to that achieved with the optimal 10 μg QS-21 control.

DETD CTL Induced by OS-21 and **CpG**/OS-21

DETD As evident from the results in FIG. 2, no lysis was observed in the absence of adjuvant, with any **CpG** dose, or with 1.25 μg of QS-21 (suboptimal dose). However, the suboptimal dose of QS-21, in combination with **CpG**, induced significant CTL (comparable to the optimal 10 μg QS-21 control). The results illustrate the positive synergism between the **CpG** and the QS-21 that was unexpected at a suboptimal dose.

DETD . . . not detectable in any groups except for the combination of 10 μg QS-21 (optimal dose) with 10 or 50 μg **CpG** and the combination of 1.25 μg QS-21 (suboptimal dose) with 50 μg **CpG**. IgG2a was not detected with any **CpG** dose used alone, with any QS-21 dose used alone, or in the unadjuvanted group.

DETD Antibody Induced by OS-21 and OS-21/**CpG** to Pneumococcal Polysaccharide Antigen

DETD . . . pneumococcal Type 14 polysaccharide plus the indicated doses of adjuvant in a total volume of 0.2 ml phosphate-buffered saline. The **immunostimulatory** motif **CpG** used in this experiment was a phosphorothioate-modified oligonucleotide 1826 with a sequence of **TCCATGACGTTCCTGACGTT** [SEQ ID NO.:2] (Chu, et al., Exp. Med. 186:1623-1631 (1997)). QS-21 was used at a dose of 1.25 μg or 10 μg. **CpG** ODN 1826 was used at a dose of only 10 μg.

DETD . . . mice in each group. After a single immunization, IgG1 titers were 66 fold higher for the 10 μg QS-21/10 μg **CpG** combination than for QS-21 alone and were 43 fold higher than for **CpG** alone (FIG. 4). IgG2a titers were 11 fold higher for the 10 μg QS-21/**CpG** combination than for either QS-21 alone or **CpG** alone (FIG. 5). IgG3 titers were 85 fold higher for the 10 μg QS-21/**CpG** combination than for QS-21 alone and were 95 fold higher than for **CpG** alone (FIG. 6).

DETD After two immunizations, IgG1 titers were 46 fold higher for the 10 μg QS-21/**CpG** combination than for QS-21 alone and were 444 fold higher than for **CpG** alone (FIG. 7). IgG2a titers were 476 fold higher for the 10 μg QS-21/**CpG** combination than for QS-21 alone and were 127 fold higher than for **CpG** alone (FIG. 5). IgG3 titers were 67 fold higher for the 10 μg QS-21/**CpG** combination than for QS-21 alone and were 243 fold higher than for **CpG** alone (FIG. 9). The enhancement of these titers shows that this is a positive synergistic effect and is not simply. . . effect of combining these two adjuvants. In addition, the combination of low doses of QS-21 (1.25 μg) with 10 μg **CpG** also produced IgG1 and IgG3 titers after two immunizations that were higher than those produced by either 1.25 μg QS-21 alone, 10 μg QS-21 alone, or 10 μg **CpG** alone.

. . . composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide is not a part of a DNA vaccine vector, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

5. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises more than one unmethylated **CpG** dinucleotide.

6. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate,. . .

7. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

8. The immune adjuvant composition as claimed in claim 1, wherein the **immunostimulatory** oligonucleotide comprises a **CpG** motif having the formula 5'X_1CG$X_2$3', wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

9. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1).

. . . composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the saponin is substantially pure, and the saponin is QS-7, QS-17 or QS-18, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

. . . composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

13. The immune adjuvant composition as claimed in claim 12, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

. . . composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide comprises TCTCCCAGCGTFCGCCAT (SEQ ID NO:1), and, wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

. . . composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide comprises **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2), and

wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

. . . composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the **immunostimulatory** oligonucleotide is 4-40 bases in length, and wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

. . . activity, wherein the saponin (i) is derived from Quillaja saponaria and (ii) is a chemically modified saponin; and (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide, wherein the saponin and **immunostimulatory** oligonucleotide have a synergistic adjuvant effect.

25. The immune adjuvant composition as claimed in claim 1 or 4, wherein the **immunostimulatory** oligonucleotide comprises **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

. . . 30. The method as claimed in any of claims 11, 14, 15, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises more than one unmethylated **CpG** dinucleotide.

31. The method as claimed in any of claims 11, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate,. . .
32. The method as claimed in any of claims 11, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

. . . 33. The method as claimed in any of claims 11, 14, 15, 17, 19, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises a **CpG** motif having the formula 5'X_{1CGX23}', wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

34. The method as claimed in any of claims 11, 14, 15, 21, 23, or 26, wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

. . . vaccine composition comprising (a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from Quillaja saponaria; (b) an **immunostimulatory** oligonucleotide comprising at least one unmethylated **CpG** dinucleotide; and (c) a nucleic acid molecule comprising a nucleotide sequence encoding an antigen, wherein the nucleotide sequence is operatively linked to a promoter, wherein the **immunostimulatory** oligonucleotide is not a part of the nucleic acid molecule comprising the nucleotide sequence encoding the antigen.

42. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises more than one unmethylated **CpG** dinucleotide.

43. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate,. . .
44. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises at least one phosphorothioate modified nucleotide.

45. The vaccine composition as claimed in claim 38, wherein the **immunostimulatory** oligonucleotide comprises a **CpG** motif having the formula 5'X_{1CGX23}', wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

46. The vaccine composition as claimed in claim 38 or 41, wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

. . . 14, 15, 21, 23, and 26, wherein the saponin is substantially pure, wherein the saponin is QS-21, and wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

51. The immune adjuvant composition as claimed in claim 12, 20 or 22, wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

54. The immune adjuvant composition as claimed in claim 53, wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

57. The immune adjuvant composition as claimed in claim 20 or 56, wherein the **immunostimulatory** oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate,. . .
58. The immune adjuvant composition as claimed in claim 56, wherein the **immunostimulatory** oligonucleotide comprises **TCTCCCAGCGTGCGCCAT** (SEQ ID NO:1) or **TCCATGACGTTCCTGACGTT** (SEQ ID NO:2).

L17 ANSWER 2 OF 8 USPATFULL on STN
2003:309071 Method of treating cancer using **immunostimulatory** oligonucleotides

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US 6653292 B1 20031125
APPLICATION: US 1999-337619 19990621 (9)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1. A method for increasing the responsiveness of a cancer cell to a cancer therapy using an **immunostimulatory** nucleic acid as compared to the absence of the **immunostimulatory** nucleic acid, comprising: administering to a subject having a cancer an effective amount for increasing the responsiveness of a cancer cell to a cancer therapy of an **immunostimulatory** nucleic acid, comprising:
5'$X_{1X2CGX3X43}$' wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides, and wherein the sequence is not palindromic.

2. The method of claim 1, further comprising administering a chemotherapeutic agent.

3. The method of claim 1, further comprising administering a cancer immunotherapeutic agent.

4. The method of claim 1, wherein the cancer is brain cancer.

5. The method of claim 1, wherein the cancer is lung cancer.

6. The method of claim 1, wherein the cancer is ovary cancer.

7. The method of claim 1, wherein the cancer is breast cancer.

8. The method of claim 1, wherein the cancer is prostate cancer.

9. The method of claim 1, wherein the cancer is colon cancer.

10. The method of claim 1, wherein the cancer is leukemia.

11. The method of claim 1, wherein the cancer is carcinoma.

12. The method of claim 1, wherein the cancer is sarcoma.

13. The method of claim 1, wherein at least one nucleotide has a phosphate backbone modification.

14. The method of claim 13, wherein the phosphate backbone modification is a phosphorothioate or phosphorodithioate modification.

15. The method of claim 14, wherein the nucleic acid backbone includes the phosphate backbone modification on the 5' inter-nucleotide linkages.

16. The method of claim 14, wherein the nucleic acid backbone includes the phosphate backbone modification on the 3' inter-nucleotide linkages.

17. The method of claim 1, wherein the oligonucleotide has 8 to 100 nucleotides.

18. The method of claim 1, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

19. The method of claim 1, wherein X_{1X2} are GpA and X_{3X4} are TpT.

20. The method of claim 1, wherein X_{1X2} are both purines and X_{3X4} are both pyrimidines.

21. The method of claim 1, wherein X_{1X2} are GpA and X_{3X4} are both pyrimidines.

22. The method of claim 1, wherein the oligonucleotide is 8 to 40 nucleotides in length.

23. The method of claim 1, wherein the oligonucleotide is isolated.

24. The method of claim 1, wherein the oligonucleotide is a synthetic oligonucleotide.

25. A method for enhancing recovery of bone marrow using an **immunostimulatory** nucleic acid as compared to the absence of the **immunostimulatory** nucleic acid in a subject undergoing or having undergone cancer therapy, comprising: administering to a subject undergoing or having undergone cancer therapy which damages the bone marrow an effective amount for enhancing the recovery of bone marrow of an **immunostimulatory** nucleic acid, comprising: $5'X_{1X2CGX3X43}'$ wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides.

26. The method of claim 25, wherein at least one nucleotide has a phosphate backbone modification.

27. The method of claim 26, wherein the phosphate backbone modification is a phosphorothioate or phosphorodithioate modification.

28. The method of claim 25, wherein the oligonucleotide has 8 to 100 nucleotides.

29. The method of claim 25, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

30. In a method for stimulating an immune response in a subject having a cancer, the method of the type involving antigen dependent cellular cytotoxicity (ADCC), the improvement comprising: administering to the subject an **immunostimulatory** nucleic acid, comprising: $5'X_{1X2CGX3X43}'$ wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides.

31. The method of claim 30, wherein at least one nucleotide has a phosphate backbone modification.

32. The method of claim 30, wherein the oligonucleotide has 8 to 100 nucleotides.

33. The method of claim 32, wherein the phosphate backbone modification is a phosphorothioate or phosphorodithioate modification.

34. The method of claim 32, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

35. The method of claim 30, wherein 5' $X_{1X2CGX3X4}$ 3' is not palindromic.

36. A method for treating or preventing cancer, comprising: administering to a subject having a cancer an effective amount for treating or preventing cancer of an **immunostimulatory** nucleic acid, comprising: $5'X_{1X2CGX3X43}'$ wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides,

and wherein the sequence is not palindromic.

37. The method of claim 36, further comprising administering a chemotherapeutic agent.

38. The method of claim 36, further comprising administering a cancer immunotherapeutic agent.

39. The method of claim 36, wherein the cancer is brain cancer.

40. The method of claim 36, wherein the cancer is lung cancer.

41. The method of claim 36, wherein the cancer is ovarian cancer.

42. The method of claim 36, wherein the cancer is breast cancer.

43. The method of claim 36, wherein the cancer is prostate cancer.

44. The method of claim 36, wherein the cancer is colon cancer.

45. The method of claim 36, wherein the cancer is leukemia.

46. The method of claim 36, wherein the cancer is carcinoma.

47. The method of claim 36, wherein the cancer is sarcoma.

48. The method of claim 36, wherein at least one nucleotide has a phosphate backbone modification.

49. The method of claim 48, wherein the phosphate backbone modification is a phosphorothioate or phosphorodithioate modification.

50. The method of claim 49, wherein the nucleic acid backbone includes the phosphate backbone modification on the 5' inter-nucleotide linkages.

51. The method of claim 49, wherein the nucleic acid backbone includes the phosphate backbone modification on the 3' inter-nucleotide linkages.

52. The method of claim 36, wherein the oligonucleotide has 8 to 100 nucleotides.

53. The method of claim 36, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

54. The method of claim 36, wherein X_{1X2} are GpA and X_{3X4} are TpT.

55. The method of claim 36, wherein X_{1X2} are both purines and X_{3X4} are both pyrimidines.

56. The method of claim 36, wherein X_{1X2} are GpA and X_{3X4} are both pyrimidines.

57. The method of claim 36, wherein the oligonucleotide is 8 to 40 nucleotides in length.

TI Method of treating cancer using **immunostimulatory** oligonucleotides
AI US 1999-337619 19990621 (9)
AB Nucleic acid sequences containing unmethylated **CpG** dinucleotides that modulate an immune response including stimulating a Th1 pattern of immune activation, cytokine production, NK lytic activity, and. . .
SUMM . . . present invention relates generally to oligonucleotides and more specifically to oligonucleotides which have a sequence including at least one unmethylated **CpG** dinucleotide which are **immunostimulatory**.
SUMM . . . CRE, the consensus form of which is the unmethylated sequence TGACGTC (SEQ. ID. No. 103) (binding is abolished if the **CpG** is methylated) (Iguchi-Ariga, S. M. M., and W. Schaffner: "**CpG** methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA (SEQ. ID. No.104) abolishes specific factor binding as well as transcriptional activation." Genese. . .
SUMM The present invention is based on the finding that certain nucleic acids containing unmethylated cytosine-guanine (**CpG**) dinucleotides activate lymphocytes in a subject and redirect a subject's immune response from a Th2 to a Th1 (e.g., by. . . to produce Th1 cytokines, including IL-12, IFN-γ and GM-CSF). Based on this finding, the invention

features, in one aspect, novel **immunostimulatory** nucleic acid compositions.

SUMM In one embodiment, the invention provides an isolated **immunostimulatory** nucleic acid sequence containing a **CpG** motif represented by the formula:

SUMM In another embodiment, the invention provides an isolated **immunostimulatory** nucleic acid sequence contains a **CpG** motif represented by the formula:

SUMM In another embodiment, autoimmune disorders are treated by inhibiting a subject's response to **CpG** mediated leukocyte activation. The invention provides administration of inhibitors of endosomal acidification such as bafilomycin a, chloroquine, and monensin to. . .

DRWD FIG. 1B. Control phosphodiester oligodeoxynucleotide (ODN) 5' ATGGAAGGTCCAGTGTTCTC 3' (SEQ ID NO:1 14) (.box-solid.) and two phosphodiester **CpG** ODN 5' ATCGACCTACGTGCGTTCTC 3' (SEQ ID NO:2) (.diamond-solid.) and 5' TCCATAACGTTCCTGATGCT 3' (SEQ ID NO:3) (.circle-solid.).

DRWD FIG. 1C. Control phosphorothioate ODN 5' GCTAGATGTTAGCGT 3' (SEQ ID NO:4) (.box-solid.) and two phosphorothioate **CpG** ODN 5' GAGAACGTCGACCTTCGAT 3' (SEQ ID NO: 5) (.box-solid.) and 5' GCATGACGTTGAGCT 3' (SEQ ID NO:6) (.circle-solid.). Data present the. . .

DRWD FIG. 2 is a graph plotting IL-6 production induced by **CpG** DNA in vivo as determined 1-8 hrs after injection. Data represent the mean for duplicate analyses of sera from two mice. BALB/c mice (two mice/group) were inject iv. with 100 µl of PBS (.quadrature.) of 200 µg of **CpG** phosphorothioate ODN 5' TCCATGACGTTCCTGATGCT 3' (SEQ ID NO:7) (.box-solid.) or non-**CpG** phosphorothioate 5' TCCATGAGCTTCCTGAGTCT 3' (SEQ ID NO: 8) (.diamond-solid.).

DRWD . . . periods after in vivo stimulation of BALB/c mice (two mice/group) injected iv with 100 µl of PBS, 200 µl of **CpG** phosphorothioate ODN 5' TCCATGACGTTCCTGATGCT 3' (SEQ ID NO: 7) or non-**CpG** phosphorothioate ODN 5' TCCATGAGCTTCCTGAGTCT 3' (SEQ ID NO: 8).

DRWD FIG. 4A is a graph plotting dose-dependent inhibition of **CpG**-induced IgM production by anti-IL-6. Splenic B-cells from DBA/2 mice were stimulated with **CpG** ODN 5' TCCAAGACGTTCCTGATGCT 3' (SEQ ID NO:9) in the presence of the indicated concentrations of neutralizing anti-IL-6 (.diamond-solid.) or isotype control Ab (.circle-solid.) and IgM levels in culture supernatants determined by ELISA. In the absence of **CpG** ODN, the anti-IL-6 Ab had no effect on IgM secretion (.box-solid.).

DRWD FIG. 4B is a graph plotting the stimulation index of **CpG**-induced splenic B cells cultured with anti-IL-6 and **CpG** S--ODN 5' TCCATGACGTTCCTGATGCT 3' (SEQ ID NO:7) (.diamond-solid.) or anti-IL-6 antibody only (.box-solid.). Data present the mean±standard deviation of triplicates.

DRWD . . . cells transfected with a promoter-less CAT construct (pCAT), positive control plasmid (RSV), or IL-6 promoter-CAT construct alone or cultured with **CpG** 5' TCCATGACGTTCCTGATGCT 3' (SEQ ID NO: 7) or non-**CpG** 5' TCCATGAGCTTCCTGAGTCT 3' (SEQ ID NO: 8) phosphorothioate ODN at the indicated concentrations. Data present the mean of triplicates.

DRWD FIG. 6 is a schematic overview of the immune effects of the **immunostimulatory** unmethylated **CpG** containing nucleic acids, which can directly activate both B cells and monocytic cells (including macrophages and dendritic cells) as shown. The **immunostimulatory** oligonucleotides do not directly activate purified NK cells, but render them competent to respond to IL-12 with a marked increase in their IFN-γ secretion by NK cells, the **immunostimulatory** nucleic acids promote a Th1 type immune response. No direct activation of proliferation of cytokine secretion by highly purified T cells has been found. However, the induction of Th1 cytokine secretion by the **immunostimulatory** oligonucleotides promotes the development of a cytotoxic lymphocyte response.

DRWD FIG. 7 is an autoradiograph showing NFκB mRNA induction in monocytes treated with E. coli (EC) DNA (containing unmethylated **CpG** motifs), control (CT) DNA (containing no unmethylated **CpG** motifs) and lipopolysaccharide (LPS) at various measured times, 15 and 30 minutes after contact.

DRWD . . . the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the **CpG** oligo (**TCCATGACGTTCCTGACGTT** SEQ ID NO: 10) also showed an increase in the level of reactive oxygen species such that more than 50%. . .

DRWD . . . have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with **CpG** DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and. . .

DRWD . . . egg antigen "SEA" (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given **CpG** oligo (SEQ ID NO: 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation. . .

DRWD . . . and subsequently inhale SEA (open circle), many eosinophils are present in the lungs. However, when the mice are initially given **CpG** oligo (SEQ ID NO: 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation. . .

DRWD . . . inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a **CpG** oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes. . .

DRWD . . . or SEQ ID NO. 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can actually redirect the cytokine response of the lung to production of IL-12, indicating a Th1 type of immune. . .

DRWD . . . or SEQ ID NO: 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can also redirect the cytokine response of the lung to production of IFN-g, indicating a Th1 type of immune. . .

DETD An "**immunostimulatory** nucleic acid molecule" refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (i.e., "**CpG** DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates (e.g., has a motogenic effect on, or induces or increases cytokine expression by) a vertebrate lymphocyte. An **immunostimulatory** nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have. . .

DETD In one preferred embodiment, the invention provides an isolated **immunostimulatory** nucleic acid sequence containing a **CpG** motif represented by the formula:

DETD In another embodiment the invention provides an isolated **immunostimulatory** nucleic acid sequence contains a **CpG** motif represented by the formula:

DETD Preferably, the **immunostimulatory** nucleic acid sequences of the invention include X_1X_2 selected from the group consisting of GpT, GpG, GpA and ApA and. . . selected from the group consisting of TpT, CpT and GpT (see for example, Table 5). For facilitating uptake into cells, **CpG** containing **immunostimulatory** nucleic acid molecules are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size (even many kb long) are **immunostimulatory** if sufficient **immunostimulatory** motifs are present, since such larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not. . . or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus mitogenic **CpG** motif is not a palindrome. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, where the oligonucleotide incorporates a phosphate backbone. . .

DETD Preferably the **immunostimulatory CpG** DNA is in the range of between 8 to 30 bases in size when it is an oligonucleotide. Alternatively, **CpG** dinucleotides can be produced on a large scale in plasmids, which after being administered to a subject are degraded into oligonucleotides. Preferred **immunostimulatory** nucleic acid molecules (e.g., for use in increasing the effectiveness of a vaccine or to treat an immune system deficiency. . .

DETD . . . useful as an adjuvant for use during antibody production in a mammal. Specific, but non-limiting examples of such sequences include: **TCCATGACGTTCCTGACGTT** (SEQ ID NO: 10), GTCGTT (SEQ. ID. NO: 57), GTCGCT (SEQ. ID. NO.58), TGTCGCT (SEQ. ID. NO: 101) and TGTCGTT. . . symptoms of an asthmatic disorder by redirecting a subject's immune response from Th2 to Th 1. An exemplary sequence includes **TCCATGACGTTCCTGACGTT** (SEQ ID NO: 10).

DETD The stimulation index of a particular **immunostimulatory CpG** DNA can be tested in various immune cell assays. Preferably, the stimulation index of the **immunostimulatory CpG** DNA with regard to B-cell proliferation is at least about 5, preferably at least about 10, more preferably at least. . . treat an immune system deficiency by stimulating a cell-mediated (local) immune response in a subject, it is important that the **immunostimulatory CpG** DNA be capable of effectively inducing cytokine secretion by monocytic cells and/or Natural Killer (NK) cell lytic activity.

DETD Preferred **immunostimulatory CpG** nucleic acids should effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml. . . IL-6, 200 pg/ml IL-12, depending on the therapeutic indication, as determined by the assays described in Example 12. Other preferred **immunostimulatory CpG** DNAs should effect at least about 10%, more preferably at least about 15% and most preferably at least about 20%. . .

DETD . . . in vivo degradation (e.g., via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated **CpG** containing nucleic acid molecules that are tens to hundreds of kbs long are relatively resistant to in vivo degradation.

For shorter **immunostimulatory** nucleic acid molecules, secondary structure can stabilize and increase their effect. For example, if the 3' end of a nucleic. . .

DETD . . . acid molecules (including phosphorodithioate-modified) can increase the extent of immune stimulation of the nucleic acid molecule, which contains an unmethylated **CpG** dinucleotide as shown herein. International Patent Application Publication Number WO 95/26204 entitled "Immune Stimulation By Phosphorothioate Oligonucleotide Analogs" also reports on the non-sequence specific **immunostimulatory** effect of phosphorothioate modified oligonucleotides. As reported herein, unmethylated **CpG** containing nucleic acid molecules having a phosphorothioate backbone have been found to preferentially activate B-cell activity, while unmethylated **CpG** containing nucleic acid molecules having a phosphodiester backbone have been found to preferentially activate monocytic (macrophages, dendritic cells and monocytes) and NK cells. Phosphorothioate **CpG** oligonucleotides with preferred human motifs are also strong activators of monocytic and NK cells.

DETD Certain Unmethylated **CpG** Containing Nucleic Acids Have B Cell Stimulatory Activity as Shown in vitro and in vivo

DETD . . . the other nonstimulatory control ODN. In comparing these sequences, it was discovered that all of the four stimulatory ODN contained **CpG** dinucleotides that were in a different sequence context from the nonstimulatory control.

DETD To determine whether the **CpG** motif present in the stimulatory ODN was responsible for the observed stimulation, over 300 ODN ranging in length from 5 to 42 bases that contained methylated, unmethylated, or no **CpG** dinucleotides in various sequence contexts were synthesized. These ODNs, including the two original "controls" (ODN 1 and 2) and two. . . then examined for in vitro effects on spleen cells (representative sequences are listed in Table 1). Several ODN that contained **CpG** dinucleotides induced B cell activation and IgM secretion; the magnitude of this stimulation typically could be increased by adding more **CpG** dinucleotides (Table 1; compare ODN 2 to 2a or 3D to 3Da and 3Db). Stimulation did not appear to result. . .

DETD Mitogenic ODN sequences uniformly became nonstimulatory if the **CpG** dinucleotide was mutated (Table 1; compare ODN 1 to 1a; 3D to 3Dc; 3M to 3Ma; and 4 to 4a) or if the cytosine of the **CpG** dinucleotide was replaced by 5-methylcytosine (Table 1; ODN 1b, 2b, 3Dd, and 3Mb). Partial methylation of **CpG** motifs caused a partial loss of stimulatory effect (compare 2a to 2c, Table 1). In contrast, methylation of other cytosines did not reduce ODN activity (ODN 1c, 2d, 3De and 3Mc). These data confirmed that **CpG** motif is the essential element present in ODN that activate B cells.

DETD In the course of these studies, it became clear that the bases flanking the **CpG** dinucleotide played an important role in determining the murine B cell activation induced by an ODN The optimal stimulatory motif was determined to consist of a **CpG** flanked by two 5' purines (preferably a GpA dinucleotide) and two 3' pyrimidines (preferably a TpT or TpC dinucleotide). Mutations of ODN to bring the **CpG** motif closer to this ideal improved stimulation (e.g., Table 1, compare ODN 2 to 2e; 3M to 3Md) while mutations. . . Table 1, compare ODN 3D to 3Df; 4 to 4b, 4c and 4d). On the other hand, mutations outside the **CpG** motif did not reduce stimulation (e.g., Table 1, compare ODN to 1d; 3D to 3Dg; 3M to 3Me). For activation. . .

DETD . . . 10 As. The effect of the G-rich ends is cis; addition of an ODN with poly G ends but no **CpG** motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated **CpG** were found to be more **immunostimulatory**.

DETD . . . from at least 3 separate experiments, and are compared to wells cultured with no added ODN. ND = not done. **CpG** dinucleotides are underlined. Dots indicate identity; dashes indicate deletions. Z = 5 methyl cytosine.

DETD

TABLE 2

Identification of the optimal **CpG** motif for Murine IL-6 production and B cell activation.

ODN	SEQUENCE (5'-3')	CH12.LX	SPLENIC B CELL SI[b]	IgM (ng/ml)[c]
		IL-6 (pg/ml)[a]		
512.	. .	3534 ± 217		
1708 (SEQ ID No:40	.....CA..TG.....	ND	59 ± 3	1.5 ± 0.1 466 ± 109

Dots indicate identity; **CpG** dinucleotides are underlined; ND = not done

[a]The experiment was done at least three times with similar results. The level. . . both CH12.LX and splenic B cells was ≤10 pg/ml. The IgM level of unstimulated culture was 547 ± 82 ng/ml. **CpG**

dinucleotides are underlined and dots indicate identity. Cells were stimulated with 20 μM of various **CpG** O-ODN. Data present the mean ± SD of triplicates.

b[3H] Uridine uptake was indicated as a fold increase (SI: stimulation. . .

DETD . . . the subsequent fall in stimulation when purified B cells with or without anti-IgM (at a submitogenic dose) were cultured with **CpG** ODN, proliferation was found to synergistically increase about 10-fold by the two mitogens in combination after 48 hours. The magnitude. . .

DETD Cell cycle analysis was used to determine the proportion of B cells activated by **CpG**-ODN. **CpG**-ODN induced cycling in more than 95% of B cells. Splenic B lymphocytes sorted by flow cytometry into CD23- (marginal zone). . . as were both resting and activated populations of B cells isolated by fractionation over Percoll gradients. These studies demonstrated that **CpG**-ODN induced essentially all B cells to enter the cell cycle.

DETD **Immunostimulatory** Nucleic Acid Molecules Block Murine B Cell Apoptosis

DETD . . . are rescued from this growth arrest by certain stimuli such as LPS and by the CD40 ligand. ODN containing the **CpG** motif were also found to protect WEHI-231 from anti-IgM induced growth arrest, indicating that accessory cell populations are not required for the effect. Subsequent work indicates that **CpG** ODN induce Bcl-x and myc expression, which may account for the protection from apoptosis. Also, **CpG** nucleic acids have been found to block apoptosis in human cells. This inhibition of apoptosis is important, since it should enhance and prolong immune activation by **CpG** DNA.

DETD Identification of the Optimal **CpG** Motif for Induction of Murine IL-6 and IgM Secretion and B Cell Proliferation

DETD To evaluate whether the optimal B cell stimulatory **CpG** motif was identical with the optimal **CpG** motif for IL-6 secretion, a panel of ODN in which the bases flanking the **CpG** dinucleotide were progressively substituted was studied. This ODN panel was analyzed for effects on B cell proliferation, Ig production, and. . . using both splenic B cells and CH12.LX cells. As shown in Table 2, the optimal stimulatory motif contains an unrmethylated **CpG** flanked by two 5' purines and two 3' pyrimidines. Generally a mutation of either 5' purines to C were especially. . . had less marked effects. Based on analyses of these and scores of other ODN, it was determined that the optimal **CpG** motif for induction of IL-6 secretion is TGACGTT (SEQ. ID. NO: 108), which is identical with the optimal mitogenic and IgM-inducing **CpG** motif (Table 2). This motif was more stimulatory than any of the palindrome containing sequences studied (1639, 1707 and 1708).

DETD Induction of Murine Cytokine Secretion by **CpG** Motifs in Bacterial DNA or Oligonucleotides

DETD As described in Example 9, the amount of IL-6 secreted by spleen cells after **CpG** DNA stimulation was measured by ELISA. T cell depleted spleen cell cultures rather than whole spleen cells were used for in vitro studies following preliminary studies showing that T cells contribute little or nothing to the IL-6 produced by **CpG** DNA-stimulated spleen cells. As shown in Table 3, IL-6 production was markedly increased in cells cultured with E. coli DNA. . . response to bacterial DNA. To analyze whether the IL-6 secretion induced by E. coli DNA was mediated by the unmethylated **CpG** dinucleotides in bacterial DNA, methylated E. coli DNA and a panel of synthetic ODN were examined. As shown in Table 3, **CpG** ODN significantly induced IL-6 secretion (ODN 5a, 5b, 5c) while **CpG** methylated E. coli DNA, or ODN containing methylated **CpG** (ODN 5f) or no **CpG** (ODN 5d) did not. Changes at sites other than **CpG** dinucleotides (ODN 5b) or methylation of other cytosines (ODN 5g) did not reduce the effect of **CpG** ODN. Methylation of a single **CpG** in an ODN with three CpGs resulted in a partial reduction in the stimulation (compare ODN 5c to 5e; Table. . .

DETD

TABLE 3

Induction of Murine IL-6 secretion by **CpG** motifs in bacterial DNA or oligonucleotides.

Treatment	IL-6 (pg/ml)
calf thymus DNA	≤10
calf thymus DNA + DNase	≤10
E. coli DNA	1169.5 ± 94.1
E. coli DNA + DNase	≤10
CpG methylated E. coli DNA	≤10
LPS	280.1 ± 17.1
Media (no DNA)	≤10
5a SEQ. ID. ATGGACTCTCCAGCGTTCTC	1096.4 ± 372.0

No:115

5b. . . or without enzyme treatment, or LPS (10 µg/ml) for 24 hr. Data represent the mean (pg/ml) ± SD of triplicates. **CpG** dinucleotides are underlined and dots indicate identity. Z indicates 5-methylcytosine.

DETD **CpG** Motifs can be Used as an Artificial Adjuvant

DETD . . . more acceptable side effects has led to the production of new synthetic adjuvants. The present invention provides the sequence 1826 **TCCATGACGTTCCTGACGTT** (SEQ ID NO: 10), which is an adjuvant including **CpG** containing nucleic acids. The sequence is a strong immune activating sequence and is a superb adjuvant, with efficacy comparable or. . .

DETD Titration of Induction of Murine IL-6 Secretion by **CpG** Motifs

DETD Bacterial DNA and **CpG** ODN induced IL-6 production in T cell depleted murine spleen cells in a dose-dependent manner, but vertebrate DNA and non-**CpG** ODN did not (FIG. 1). IL-6 production plateaued at approximately 50 µg/ml of bacterial DNA or 40 µM of **CpG** O--ODN. The maximum levels of IL-6 induced by bacterial DNA and **CpG** ODN were 1-1.5 ng/ml and 2-4 ng/ml respectively. These levels were significantly greater than those seen-after stimulation by LPS (0.35 ng/ml) (FIG. 1A). To evaluate whether **CpG** ODN with a nuclease-resistant DNA backbone would also induce IL-6 production, S--ODN were added to T cell depleted murine spleen cells. **CpG** S--ODN also induced IL-6 production in a dose-dependent manner to approximately the same level as **CpG** O--ODN while non-**CpG** S--ODN failed to induce IL-6 (FIG. 1C). **CpG** S--ODN at a concentration of 0.05 µM could induce maximal IL-6 production in these cells. This result indicted that the nuclease-resistant DNA backbone modification retains the sequence specific ability of **CpG** DNA to induce IL-6 secretion and that **CpG** S--ODN are more than 80-fold more potent than **CpG** O--ODN in this assay system.

DETD Induction of Murine IL-6 Murine by **CpG** DNA in vivo

DETD To evaluate the ability of bacterial DNA and **CpG** S--ODN to induce Il-6 secretion in vivo, BALB/c mice were injected iv. with 100 µg of E. coli DNA, calf thymus DNA, or **CpG** or non-stimulatory S--ODN and bled 2 hr after stimulation. The level of IL-6 in the sera from the E. coli. . . 13 ng/ml while IL-6 was not detected in the sera from calf thymus DNA or PBS injected groups (Table 4). **CpG** S--ODN also induced IL-6 secretion in vivo. The IL-6 level in the sera from **CpG** S--ODN injected groups was approximately 20 ng/ml. In contrast, IL-6 was not detected in the sera from non-stimulatory S--ODN stimulated. . .

DETD

TABLE 4

Secretion of Murine IL-6 induced by **CpG** DNA stimulation in vivo.

Stimulant	IL-6 (pg/ml)
PBS	<50
E. coli DNA	13858 ± 3143
Calf Thymus DNA	<50
CpG S-ODN	20715 ± 606
non-**CpG** S-ODN	<50

Mice (2 mice/group) were i.v. injected with 100 µl of PBS, 200 µl of E. coli DNA or calf thymus DNA, or 500 µg of **CpG** S-ODN or non-**CpG** control S-ODN. Mice were bled 2 hr after injection and 1:10 dilution of each serum was analyzed by IL-6 ELISA. Sensitivity limit of IL-6 ELISA was 5 pg/ml. Sequences of the **CpG** S-ODN is 5'GCATGACGTTGAGCT3'(SEQ. ID. No:6) and of the non-stimulatory S-ODN is 5'GCTAGATGTTAGCGT3'(SEQ. ID. No:4). Note that although there is a **CpG** in sequence 48, # it is too close to the 3' end to effect stimulation, as explained herein. Data. . .

DETD Kinetics of Murine IL-6 Secretion After Stimulation by **CpG** Motifs in vivo

DETD To evaluate the kinetics of induction of IL-6 secretion by **CpG** DNA n vivo, BALB/c mice were injected iv. with **CpG** or control non-**CpG** S--ODN. Serum IL-6 levels were significantly increased within 1 hr and peaked at 2 hr to a level of approximately 9 ng/ml in the **CpG** S--ODN injected group (FIG. 2). Il-6 protein in sera rapidly decreased after 4 hr and returned to basal level by 12 hr after stimulation. In contrast to **CpG** DNA stimulated groups, no significant increase of IL-6 was observed in the sera from the non-stimulatory S--ODN or PBS injected. .

DETD Tissue Distribution and Kinetics of IL-6, mRNA Expression Induced by **CpG** Motifs in vivo

DETD As shown in FIG. 2, the level of serum IL-6 increased rapidly after **CpG** DNA stimulation. To investigate the possible tissue origin of this serum IL-6, and the kinetics of IL-6 gene expression in vivo after **CpG** DNA stimulation, BALB/c mice were injected iv with **CpG** or non-**CpG** S--ODN and RNA was extracted from liver, spleen, thymus, and bone marrow

at various time points after stimulation. As shown. . . FIG. 3A, the level of IL-6 mRNA in liver, spleen, and thymus was increased within 30 min. after injection of **CpG** S--ODN. The liver IL-6 mRNA peaked at 2 hr post-injection and rapidly decreased and reached basal level 8 hr after. . . hr post-injection and then gradually decreased (FIG. 3A). IL-6 mRNA was significantly increased in bone marrow within 1 hr after **CpG** S--ODN injection but then returned to basal level. In response to **CpG** S--ODN, liver, spleen and thymus showed more substantial increases in IL-6 mRNA expression than the bone marrow.

DETD Patterns of Murine Cytokine Expression Induced by **CpG** DNA

DETD . . . within 30 minutes and the level of IL-6 increased strikingly within 2 hours in the serum of mice injected with **CpG** ODN. Increased expression of IL-12 and interferon gamma (IFN-γ) mRNA by spleen cells was also detected within the first two. . .

DETD . . . 0 70

SEQ ID NO:39

1708 . . . CA_TG . . . 270 10 17 ND 10

SEQ ID NO:40

dots indicate identity; **CpG** dinucleotides are underlined

[1]measured by ELISA using Quantikine kits from R&D Systems (pg/ml) Cells were cultured in 10% autologous serum. . .

DETD **CpG** DNA Induces Cytokine Secretion by Human PBMC, Specifically Monocytes

DETD . . . panels of ODN used for studying mouse cytokine expression were used to determine whether human cells also are induced by **CpG** motifs to express cytokine (or proliferate), and to identify the **CpG** motif(s) responsible. Oligonucleotide 1619 (GTCGTT; SEQ. ID. NO: 57) was the best inducer of TNF-α and IFN-γ secretion, and was. . . little induction of other cytokines. Thus, it appears that human lymphocytes, like murine lymphocytes, secrete cytokines differentially in response to **CpG** dinucleotides, depending on the surrounding bases. Moreover, the motifs that stimulate murine cells best differ from those that are most effective with human cells. Certain **CpG** oligodeoxynucleotides are poor at activating human cells (oligodeoxynucleotides 1707, 1708, which contain the palindrome forming sequences GACGTC (SEQ. ID. NO:. . .

DETD . . . simply reflect a nonspecific death of all cell types. Cytokine secretion in response to E. coli (EC) DNA requires unmethylated **CpG** motifs, since it is abolished by methylation of the EC DNA (next to the bottom row, Table 6). LPS contamination. . .

DETD

TABLE 6

CpG DNA induces cytokine secretion by human PBMC

DNA	TNF-α(pg/ml)[1]	IL-6 (pg/ml)	IFN-γ (pg/ml)	RANTES (pg/ml)
EC DNA (50 μg/ml)	900	12,000	. . .	

cytokine production under these conditions was from monocytes (or other L-LME-sensitive cells).

[3]EC DNA was methylated using 2U/μg DNA of **CpG** methylase (New England Biolabs) according to the manufacturer's directions, and methylation confirmed by digestion with Hpa-II and Msp-I. As a. . .

DETD . . . cytokine production in the PBMC treated with L-LME suggested that monocytes may be responsible for cytokine production in response to **CpG** DNA. To test this hypothesis more directly, the effects of **CpG** DNA on highly purified human monocytes and macrophages was tested. As hypothesized, **CpG** DNA directly activated production of the cytokines IL-6, GM-CSF, and TNF-α by human macrophages, whereas non-**CpG** DNA did not (Table 7).

DETD

TABLE 7

CpG DNA induces cytokine expression in purified human macrophages

	IL-6 (pg/ml)	GM-CSF (pg/ml)	TNF-α(pg/ml)
Cells alone	0	0	0
CT DNA (50 μg/ml). . .			

DETD Biological Role of IL-6 in Inducing Murine IgM Production in Response to **CpG** Motifs

DETD The kinetic studies described above revealed that induction of IL-6 secretion, which occurs within 1 hr post **CpG** stimulation, precedes IgM secretion. Since the optimal **CpG** motif for ODN inducing secretion of IL-6 is the same as that for IgM (Table 2), whether the **CpG** motifs independently induce IgM and IL-6 production or whether the IgM production is dependent on prior IL-6 secretion was examined. The addition of neutralizing anti-IL-6 antibodies inhibited in vitro IgM production mediated by **CpG** ODN in a dose-dependent manner but a control antibody did not (FIG. 4A). In contrast, anti-IL-6 addition did not affect either the basal level or the **CpG**-induced B cell proliferation (FIG. 4B).

DETD Increased Transcriptional Activity of the IL-6 Promoter in Response to **CpG** DNA

DETD The increased level of IL-6 mRNA and protein after **CpG** DNA stimulation could result from transcriptional or post-transcriptional regulation. To determine if the transcriptional activity of the IL-6 promoter was unregulated in B cells cultured with **CpG** ODN, a murine B cell line, WEHI-231, which produces IL-6 in response to **CpG** DNA, was transfected with an IL-6 promoter-CAT construct (pIL-6/CAT) (Pottrats, S. T. et al, 17B-estradiol) inhibits expression of human interleukin-6-promoter-reporter constructs by a receptor-dependent mechanism. J. Clin. Invest. 93:944). CAT assays were performed after stimulation with various concentrations of **CpG** or non-**CpG** ODN. As shown in FIG. 5, **CpG** ODN induced increased CAT activity in dose-dependent manner while non-**CPG** ODN failed to induce CAT activity. This confirms that **CpG** induces the transcriptional activity of the IL-6 promoter.

DETD Dependence of B Cell Activation by **CpG** ODN on the Number of 5' and 3' Phosphorothioate Internucleotide Linkages

DETD . . . or DNA synthesis (by ^{3H} thymidine incorporation) in treated spleen cell cultures (Example 10). O--ODN (0/0 phosphorothioate modifications) bearing a **CpG** motif caused no spleen cell stimulation unless added to the cultures at concentrations of at least 10 μM (Example 10).. . .

DETD . . . result from the nuclease resistance of the former. To determine the role of ODN nuclease resistance in immune stimulation by **CpG** ODN, the stimulatory effects of chimeric ODN in which the 5' and 3' ends were rendered nuclease resistant with either. . .

DETD . . . while the S--ODN with the 3D sequence was less potent than the corresponding S--O--ODN (Example 10). In comparing the stimulatory **CpG** motifs of these two sequences, it was noted that the 3D sequence is a perfect match for the stimulatory motif in that the **CpG** is flanked by two 5' purines and two 3' pyrimidines. However, the bases immediately flanking the **CpG** in ODN 3D are not optimal; it has a 5' pyrimidine and a 3' purine. Based on further testing, it. . . for immune stimulation is more stringent for S--ODN than for S--O-- or O--ODN. S--ODN with poor matches to the optimal **CpG** motif cause little or no lymphocyte activation (e.g., Sequence 3D). However, S--ODN with good matches to the motif, most critically at the positions immediately flanking the **CpG**, are more potent than the corresponding S--O--ODN (e.g., Sequence 3M, Sequences 4 and 6), even though at higher concentrations (greater. . .

DETD The increased B cell stimulation seen with **CpG** ODN bearing S or S_2 substitutions could result from any or all of the following effects: nuclease resistance, increased cellular. . . localization. However, nuclease resistance cannot be the only explanation, since the MP--O--ODN were actually less stimulatory than the O--ODN with **CpG** motifs. Prior studies have shown that ODN uptake by lymphocytes is markedly affected by the backbone chemistry (Zhao, et al.. . .

DETD Unmethylated **CpG** Containing Oligos Have NK Cell Stimulatory Activity

DETD Experiments were conducted to determine whether **CpG** containing oligonucleotides stimulated the activity of natural killer (NK) cells in addition to B cells. As shown in Table 8, a marked induction of NK activity among spleen cells cultured with **CpG** ODN 1 and 3Dd was observed. In contrast, there was relatively on induction in effectors that had been treated with non-**CpG** control ODN.

DETD

TABLE 8

Induction Of NK Activity By **CpG** Oligodeoxynucleotides (ODN)

	% YAC-1		% 2C11	
	Specific Lysis*		Specific Lysis	
	Effector: Target		Effector: Target	
ODN	50:1	100:1	50:1	100:1
None	-1.1	-1.4	15.3	16.6
1	16.1	24.5	38.7	47.2
3Dd	17.1	27.0	37.0	40.0
non-**CpG** ODN	-1.6	-1.7	14.8	15.4

DETD Induction of NK Activity by DNA Containing **CpG** Motifs, but Not by Non-**CpG** DNA

DETD . . . depleted of B cells and human PBMC, but vertebrate DNA may be a consequence of its increased level of unmethylated **CpG** dinucleotides, the activating properties of more than 50 synthetic ODN containing unmethylated, methylated, or no **CpG** dinucleotides was tested. The results, summarized in Table 9, demonstrate that synthetic ODN can stimulate significant NK activity, as long as they contain at least one unmethylated **CpG** dinucleotide. No difference was observed in the stimulatory effects of ODN in which the **CpG** was within a palindrome (such as ODN 1585, which contains the palindrome AACGTT; SEQ. ID. NO: 105) from those ODN. . . palindromes (such as 1613 ro 1619), with the caveat that optimal stimulation was generally seem with ODN in which the

CpG was flanked by two 5' purines or a 5' GpT dinucleotide and two 3' pyrimidines. Kinetic experiments demonstrated that NK. . . of the ODN. The data indicates that the murine NK response is dependent on the prior activation of monocytes by **CpG** DNA, leading to the production of IL-12, TNF-α, and IFN-α/b (Example 11).

DETD

TABLE 9

Induction of NK Activity by DNA Containing **CpG** Motifs but not by Non-**CpG** DNA

DNA or Cytokine Added	Mouse Cells LU/10^6	Human Cells
Expt. 1 None	0.00	0.00
IL-2	16.68	15.82
E. Coli. DNA. . .	-------Z------ (SEQ ID No. 117)	0.02 ND
1619 TCCATGTCGTTCCTGATGCT (SEQ ID No. 38)		3.35
1765 --------Z---------- (SEQ ID No. 44)		0.11

CpG dinucleotides in ODN sequences are indicated by underlying; Z indicates methylcytosine. Lower case letters indicate nuclease resistant phosphorothioate modified internucleotide. . .

DETD From all of these studies, a more complete understanding of the immune effects of **CpG** DNA has been developed, which is summarized in FIG. 6.

DETD Immune activation by **CpG** motifs may depend on bases flanking the **CpG**, and the number of spacing of the CpGs present within an ODN. Although a single **CpG** in an ideal base context can be a very strong and useful immune activator, superior effects can be seen with ODN containing several CpGs with the appropriate spacing and flanking bases. For activation of murine B cells, the optimal **CpG** motif is TGACGTT (SEQ. ID. NO: 108); residues 10-17 of Seq. ID. No 70.

DETD . . . ODN sequences for stimulation of human cells by examining the effects of changing the number, spacing, and flanking bases of **CpG** dinucleotides.

DETD Identification of Phosphorothioate ODN with Optimal **CpG** Motifs for Activation of Human NK Cells

DETD . . . cellular uptake (Krieg et al., Antisense Res. Dev. 6:133, 1996.) and improved B cell stimulation if they also have a **CpG** motif. Since NK activation correlates strongly with in vivo adjuvant effects, the identification of phosphorothioate ODN that will activate human. .

DETD The effects of different phosphorothioate ODNs--containing **CpG** dinucleotides in various base contexts--on human NK activation (Table 10) were examined. ODN 1840, which contained 2 copies of the. . .. 10). To further identify additional ODNs optimal for NK activation, approximately one hundred ODN containing different numbers and spacing of **CpG** motifs, were tested with ODN1982 serving as a control. The result are shown in Table 1.

DETD . . . ODNs began with a TC or TG at the 5' end, however, this requirement was not mandatory. ODNs with internal **CpG** motifs (e.g., ODN 1840) are generally less potent stimulators than those in which a GTCGCT (SEQ. ID. NO: 58) motif. . . in which only one of the motifs had the additional of the spacing two Ts. The minimal acceptable spacing between **CpG** motifs is one nucleotide as long as the ODN has two pyrimidines (preferably T) at the 3' end (e.g., ODN. . . T also created a reasonably strong inducer of NK activity (e.g., ODN 2016). The choice of thymine (T) separating consecutive **CpG** dinucleotides is not absolute, since ODN 2002 induced appreciable NK activation despite the fact that adenine (A) separated its CpGs (i.e., CGACGTT; SEQ. ID. NO: 113). It should also be noted that ODNs containing no **CpG** (e.g., ODN 1982), runs of CpGs, or CpGs in bad sequence contents (e.g., ODN 2010) had no stimulatory effect on. . .

DETD

TABLE 10

ODN	Sequence (5'-3')	LU cells alone	0.01
1754	ACCATGGACGATCTGTTTCCCCTC		0.02 SEQ ID NO:59
1758	**TCTCCCAGCGTGCGCCAT**		0.05 SEQ ID NO:45
1761	TACCGCGTGCGACCCTCT		0.05 SEQ ID NO:60
1776	ACCATGGACGAACTGTTTCCCCTC		0.03 SEQ ID NO:61
1777	ACCATGGACGAGCTGTTTCCCCTC		0.05 SEQ ID NO:62
1778	ACCATGGACGACCTGTTTCCCCTC. . .		

DETD Table 11. Induction of NK LU by Phosphorothioate **CpG** ODN with Good Motifs

DETD

TABLE 11

Induction of NK LU by Phosphorothioate **CpG** ODN with Good Motifs
ODN[1] expt. 1 expt. 2 expt. 3
cells
alone sequence (5'-3') SEQ ID NO: 0.00 1.26 0.46

1840 TCCATGTCGTTCCTGTCGTT. . .
[2]This is the methylated version of ODN 1840; Z = 5-methyl cytosine LU is lytic units; ND = not done; **CpG** dinucleotides are underlined for clarity.
DETD Identification of Phosphorothioate ODN with Optimal **CpG** Motifs for Activation of Human B Cell Proliferation
DETD The ability of a **CpG** ODN to induce B cell proliferation is a good measure of its adjuvant potential. Indeed, ODN with strong adjuvant effects generally also induce B cell proliferation. To determine whether the optimal **CpG** ODN for inducing B cell proliferation are the same as those for inducing NK cell activity, similar panels of ODN. . .
DETD
TABLE 12

Induction of human B cell proliferation by Phosphorothioate **CpG** ODN
Stimulation Index[1]
ODN sequence (5'-3') SEQ ID NO: expt. 1 expt. 2 expt. 3 expt 4 expt. 5 expt. 6

1840. . .
DETD The ability of a **CpG** ODN to induce IL-12 secretion is a good measure of its adjuvant potential, especially in terms of its ability to. . . OIL-12 secretion from human PBMC in vitro (Table 13) was examined. These experiments showed that in some human PBMC, most **CpG** ODN could induce IL-12 secretion (e.g., expt. 1). However, other donors responded to just a few **CpG** ODN (E.g., expt. 2). ODN 2006 was a consistent inducer of IL12 secretion from most subjects (Table 13).
DETD
TABLE 13

Induction of human IL-12 secretion by
Phosphorothioate **CpG** ODN
IL-12 (pg/ml)
ODN[1] expt. 1 expt. 2
cells alone sequence (5'-3') SEQ ID NO: 0 0

1962 TCCTGTCGTTCCTTGTCGTT 52 19 0
1965 TCCTGTCGTTTTTTGTCGTT. . .
DETD As shown in FIG. 6, **CpG** DNA can directly activate highly purified B cells and monocytic cells. There are many similarities in the mechanism through which **CpG** DNA activates these cell types. For example, both require NFκB activation as explained further below.
DETD In further studies of different immune effects of **CpG** DNA, it was found that there is more than one type of CpG motif. Specifically, olio 1668, with the best mouse B cell motif, is a strong inducer of both B cell and. . .
DETD
TABLE 14

Different **CpG** motifs stimulate optimal murine B cell and NK activation
ODN Sequence B cell activation NK activation

1668 TCCATGACGTTCCTGATGCT (SEQ.ID.NO 7) 42,849 2.52
1758 **TCTCCCAGCGTGCGCCAT** (SEQ.ID.NO.45) 1,747 6.66
NONE 367 0.00

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance. Measured by H thymidine incorporation. . .
DETD Teleological Basis of **Immunostimulatory**, Nucleic Acids
DETD Vertebrate DNA is highly methylated and **CpG** dinucleotides are under represented. However, the stimulatory **CpG** motif is common in microbial genomic DNA, but quite rare in vertebrate DNA. In addition, bacterial DNA has been reported. . . P. et al., J. Immunol. 147:1759 (1991)). Experiments further described in Example 3, in which methylation of bacterial DNA with **CpG** methylase was found to abolish mitogenicity, demonstrates that the difference in **CpG** status is the cause of B cell stimulation by bacterial DNA. This data supports the following conclusion: that unmethylated **CpG** dinucleotides present within bacterial DNA are responsible for the stimulatory effects of bacterial DNA.
DETD Teleologically, it appears likely that lymphocyte activation by the **CpG** motif represents an immune defense mechanism that can thereby distinguish bacterial from host DNA. Host DNA, which would commonly be. . . regions and areas of inflammation due to apoptosis (cell death),

would generally induce little or mo lymphocyte activation due to **CpG** suppression and methylation. However, the presence of bacterial DNA containing unmethylated **CpG** motifs can cause lymphocyte activation precisely in infected anatomic regions, where it is beneficial. This novel activation pathway provides a rapid alternative to T cell dependent antigen specific B cell activation. Since the **CpG** pathway synergizes with B cell activation through the antigen receptor, B cells bearing antigen receptor specific for bacterial antigens would. . .

DETD . . . 35:647 (1992)), is likely triggered at least in part by activation of DNA-specific B cells through stimulatory signals provided by **CpG** motifs, as well as by binding of bacterial DNA to antigen receptors.

DETD . . . products released from dying bacteria that reach concentrations sufficient to directly activate many lymphocytes. Further evidence of the role of **CpG** DNA in the sepsis syndrome is described in Cowdery, J., et. al., (1996) the Journal of Immunology 156:4570-4575.

DETD Unlike antigens that trigger B cells through their surface Ig receptor, **CpG**-ODN did not induce any detectable Ca^{2+} flux, changes in protein tyrosine phosphorylation, or IP 3 generation. Flow cytometry with FITC-conjugated ODN with or without a **CpG** motif was performed as described in Zhao, Q et al., (Antisense Research and Development 3:53-66 (1993)), and showed equivalent membrane binding, cellular uptake, efflux, and intracellular localization. This suggests that there may not be cell membrane proteins specific for **CpG** ODN. Rather than acting through the cell membrane, that data suggests that unmethylated **CpG** containing oligonucleotides require cell uptake for activity: ODN covalently linked to a solid Teflon support were nonstimulatory, as were biotinylated ODN immobilized on either avidin beads or avidin coated petri dishes. **CpG** ODN conjugated to either FITC or biotin retained full mitogenic properties, indicated no stearic hindrance.

DETD Recent data indicate the involvement of the transcription factor NFkB as a direct or indirect mediator of the **CpG** effect. For example, within 15 minutes of treating B cells or monocytes with **CpG** DNA, the level of NFkB binding activity is increased (FIG. 7). However, it is not increased by DNA that does not contain **CpG** motifs. In addition, it was found that two different inhibitors of NFkB activation, PDTC and gliotoxin, completely block the lymphocyte stimulation by **CpG** DNA as measured by B cell proliferation or monocytic cell cytokine secretion, suggesting that NFkB activation is required for both. . .

DETD . . . various protein kinases, or through the generation of reactive oxygen species. No evidence for protein kinase activation induced immediately after **CpG** DNA treatment of B cells or monocytic cells have been found, and inhibitors of protein kinase A, protein kinase C, and protein tyrosine kinases had no effects on the **CpG** induced activation. k However, **CpG** DNA causes a rapid induction of the production of reactive oxygen species in both B cells and monocytic cells, as. . . reactive oxygen species completely block the induction of NFkB and the later induction of cell proliferation and cytokine secretion by **CpG** DNA.

DETD Work backwards, the next question was how **CpG** DNA leads to the generation of reactive oxygen species so quickly. Previous studies by the inventors demonstrated that oligonucleotides and. . . rapidly become acidified inside the cell. To determine whether this acidification step may be important in the mechanism through which **CpG** DNA activates reactive oxygen species, the acidification step was blocked with specific inhibitors of endosome acidification including chloroquine, monensin, and. . . the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the **CpG** oligo also showed an increase in the level of reactive oxygen species such that more than 50% of the cells became positive (Panel D). However, cells treated with an oligonucleotide with the identical sequence except that the **CpG** was switched did not show this significant increase in the level of reactive oxygen species (Panel E).

DETD . . . have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with **CpG** DNA (Panel B) but does not reduce the level of reactive oxygen species in t he cells treated with PMA. . . This demonstrates that unlike the PMA plus ionomycin, the generation of reactive oxygen species following treatment of B cells with **CpG** DNA requires that the DNA undergo an acidification step in the endosomes. This is a completely novel mechanism of leukocyte activation. Chloroquine, monensin, and bafilomycin also appear to block the activation of NFkB by **CpG** DNA as well as the subsequent proliferation and induction of cytokine secretion.

DETD Chronic Immune Activation by **CpG** DNA and Autoimmune Disorders

DETD B cell activation by **CpG** DNA synergizes with signals through the B cell receptor. This raises the possibility that DNA-specific B cells may be activated by the concurrent binding of bacterial DNA to their antigen receptor, and by the co-stimulatory **CpG**-mediated signals. In addition, **CpG** DNA induces B cells to become resistant to apoptosis, a mechanism

thought to be important for preventing immune responses to self antigens, such as DNA. Indeed, exposure to bDNA can trigger anti-DNA Ab production. Given this potential ability of **CpG** DNA to promote autoimmunity, it is therefore noteworthy that patients with the autoimmune disease systemic lupus erythematosus have persistently elevated. . . circulating plasma DNA which is enriched in hypomethylated CpGs. These findings suggest a possible role for chronic immune activation by **CpG** DNA in lupus etiopathogenesis.

DETD . . . While the therapeutic mechanism of these drugs has been unclear, they are known to inhibit endosomal acidification. Leukocyte activation by **CpG** DNA is not medicated through binding to a cell surface receptor, but requires cell uptake, which occurs via adsorptive endocytosis into an acidified chloroquine-sensitive intracellular compartment. This suggested the hypothesis that leukocyte activation by **CpG** DNA may occur in association with acidified endosomes, and might even be pH dependent. To test this hypothesis specific inhibitors of DNA acidification were applied to determine whether B cells or monocytes could respond to **CpG** DNA if endosomal acidification was prevented.

DETD The earliest leukocyte activation event that was detected in response to **CpG** DNA is the production of reactive oxygen species (ROS), which is induced within five minutes in primary spleen cells and. . . cell lines. Inhibitors of endosomal acidification including chloroquine, bafilomycin A, and monensin, which have different mechanisms of action, blocked the **CpG**-induced generation of ROS, but had no effect on ROS generation mediated by PMA, or ligation of CD40 or IgM. These. . . diverse pathways. This ROS generation is generally independent of endosomal acidification, which is required only for the ROS response to **CpG** DNA. ROS generation in response to **CpG** is not inhibited by the NFκB inhibitor gliotoxin, confirming that it is not secondary to NFκB activation.

DETD To determine whether endosomal acidification of **CpG** DNA was also required for its other immune stimulatory effects were performed. Both LPS and **CpG** DNA induce similar rapid NFκB activation, increases in proto-oncogene mRNA levels, and cytokine secretion. Activation of NFκB by DNA depended on **CpG** motifs since it was not induced by bDNA treated with **CpG** methylase, nor by ODN in which bases were switched to disrupt the CpGs. Supershift experiments using specific antibodies indicated that the activated NFκB complexes included the p50 and p65 components. Not unexpectedly, NFκB activation in LPS- or **CpG**-treated cells was accompanied by the degradation of IκBα and IκBβ. However, inhibitors of endosomal acidification selectively blocked all of the **CpG**-induced but none of the LPS-induced cellular activation events. The very low concentration of chloroquine (<10 μM) that has been determined to inhibit **CpG**-mediated leukocyte activation is noteworthy since it is well below that required for antimalarial activity and other reported immune effects (e.g., 100-1000 μM). These experiments support the role of a pH-dependent signaling mechanism in mediating the stimulatory effects of **CpG** DNA.

DETD
TABLE 15

Specific blockade of **CpG**-induced TNF-αand IL-12 expression by inhibitors of endosomal acidification or NFκB activation

Inhibitors:	Bafilomycin		Chloroquine		Monensin		NAC		TPCK		Gliotoxin	Bisgliotoxin
. . .	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	TNF-α	TNF-α
Medium	37	147	46	102	27	20	22	73	10	24	17	41
CpG	455	17,114	71	116	28	6	49	777	54	23	31	441
ODN												
LPS	901	22,485	1370	4051	1025	12418	491	4796.	.	.		

DETD . . . were cultured with or without the indicated inhibitors at the concentrations shown for 2 hr and then stimulated with the **CpG** oligodeoxynucleotide (ODN) 1826 (**TCCATGACGTTCCTGACGTT** SEQ ID NO:10) at 2 μM or LPS (10 μg/ml) for 4 hr (TNF-α) or 24 hr (IL-12) at which. . . Immunol., 157, 5394-5402 (1996); Krieg, A. M., J. Lab. Clin. Med., 128, 128-133 (1996). Cells cultured with ODN that lacked **CpG** motifs did not induce cytokine secretion. Similar specific inhibition of **CpG** responses was seen with IL-6 assays, and in experiments using primary spleen cells or the B cell lines CH12.LX and. . .

DETD Excessive immune activation by **CpG** motifs may contribute to the pathogenesis of the autoimmune disease systemic lupus erythematosus, which is associated with elevated levels of circulating hypomethylated **CpG** DNA. Chloroquine and related antimalarial compounds are effective therapeutic agents for the treatment of systemic lupus erythematosus and some other. . . mechanism of action has been obscure. Our

demonstration of the ability of extremely low concentrations of chloroquine to specifically inhibit **CpG**-mediated leukocyte activation suggests a possible new mechanism for its beneficial effect. It is noteworthy that lupus recurrences frequently are thought. . . bDNA present in infected tissues can be sufficient to induce a local inflammatory response. Together with the likely role of **CpG** DNA as a mediator of the sepsis syndrome and other diseases our studies suggest possible new therapeutic applications for the. . .

DETD **CpG**-induced ROS generation could be an incidental consequence of cell activation, or a signal that mediates this activation. The ROS scavenger N-acetyl-L-cysteine (NAC) blocks **CpG**-induced NFκB activation, cytokine production, and B cell proliferation, suggesting a casual role for ROS generation in these pathways. These data. . . gliotoxin (0.2 μg/ml). Cell aliquots were then cultured as above for 10 minutes in RPMI medium with or without a **CpG** ODN (1826) or non-**CpG** ODN (1911) at 1 μM or phorbol myristate acetate (PMA) plus ionomycin (iono). Cells were then stained with dihydrorhodamine-123 and. . . 5394-5402 (1996); Krieg, A. M, J. Lab. Clin. Med., 128, 128-133 (1996)). J1774 cells, a monocytic line, showed similar pH-dependent **CpG** induced ROS responses. In contrast, **CpG** DNA did not induce the generation of extracellular ROS, nor any detectable neutrophil ROS. The concentrations of chloroquine (and those used with the other inhibitors of endosomal acidification) prevented acidification of the internalized **CpG** DNA using fluorescein conjugated ODN as described by Tonkinson, et al., (Nucl. Acids Res. 22, 4268 (1994); A. M. Krieg,. . .

DETD While NFκB is known to be an important regulator of gene expression, it's role in the transcriptional response to **CpG** DNA was uncertain. To determine whether this NFκB activation was required for the **CpG** mediated induction of gene expression cells were activated with **CpG** DNA in the presence or absence of pyrrolidine dithiocarbamate (PDTC), an inhibitor of IκB phosphorylation. These inhibitors of NFκB activation completely blocked the **CpG**-induced expression of protooncogene and cytokine mRNA and protein, demonstrating the essential role of NFκB as a mediator of these events.. . . was cultured in the presence of calf thymus (CT), E. coli (EC), or methylated E. coli (mEC) DNA (methylated with **CpG** methylase as described) at 5 μg/ml or a **CpG** oligodeoxynucleotide (ODN 1826; Table 15) or a non-**CpG** ODN (ODN 1745; TCCATGAGCTTCCTGAGTCT, SEQ. ID. NO: 8) at 0.75 μM for 1 hr, following which the cells were lysed. . . was determined by supershifting with specific Ab to p65 and p50 (Santa Cruz Biotechnology, Santa Cruz, Calif.). Chloroquine inhibition of **CpG**-induced but not LPS-induced NFκB activation was established using J774 cells. The cells were precultured for 2 hr in the presence or absence of chloroquine (20 μg/ml) and then stimulated as above for 1 hr with either EC DNA, **CpG** ODN, non-**CpG** ODN or LPS (1 μg/ml). Similar chloroquine sensitive **CpG**-induced activation of NFkB was seen in a B cell line, WEHI-231 and primary spleen cells. These experiments were performed three. . .

DETD It was also established that **CpG**-stimulated mRNA expression requires endosomal acidification and NFkB activation in B cells and monocytes. J774 cells (2×10^6 cells/ml) were cultured for. . . stimulated with the addition of E. coli DNA (EC: 50 μg/ml), calf thymus DNA (CT: 50 μg/ml), LPS (10 μg/ml), **CpG** ODN (1826; 1 μM), or control non **CpG** ODN (1911; 1 μM) for 3 hr. WEHI-231 B cells (5×10^5 cells/ml) were cultured in the presence or absence of gliotoxin (0.1 μg/ml) or bisgliotoxin (0.1 μg/ml) for 2 hrs and then stimulated with a **CpG** ODN (1826), or control non-**CpG** ODN (1911; TCCAGGACTTTCCTCAGGTT, SEQ. ID. NO. 97) at 0.5 μM for 8 hr. In both cases, cells were harvested and. . .

DETD The results indicate that leukocytes respond to **CpG** DNA through a novel pathway involving the pH-dependent generation of intracellular ROS. The pH dependent step may be the transport or processing of the **CpG** DNA, the ROS generation, or some other event. ROS are widely thought to be second messengers in signaling pathways in. . .

DETD Presumably, there is a protein in or near the endosomes that specifically recognizes DNA containing **CpG** motifs and leads to the generation of reactive oxygen species. To detect any protein in the cell cytoplasm that may specifically bind **CpG** DNA, electrophoretic mobility shift assays (EMSA) were used with 5' radioactively labeled oligonucleotides with or without **CpG** motifs. A band was found that appears to represent a protein binding specifically to single stranded oligonucleotides that have **CpG** motifs, but not to oligonucleotides that lack **CpG** motifs or to oligonucleotides in which the **CpG** motif has been methylated. This binding activity is blocked if excess of oligonucleotides that contain the NFkB binding site was. . . suggests that an NFkB or related protein is a component of a protein or protein complex that binds the stimulatory **CpG** oligonucleotides.

DETD . . . points where NFkB was strongly activated. These data therefore do not provide proof the NFkB proteins actually bind to the **CpG**

nucleic acids, but rather that the proteins are required in some way for the **CpG** activity. It is possible that a CREB/ATF or related protein may interact in some way with NFkB proteins or other proteins thus explaining the remarkable similarity in the binding motifs for CREB proteins and the optimal **CpG** motif. It remains possible that the oligos bind to a CREB/ATF or related protein, and that this leads to NFkB. . .

DETD Alternatively, it is very possible that the **CpG** nucleic acids may bind to one of the TRAF proteins that bind to the cytoplasmic region of CD40 and mediate. . .

DETD Method for Making **Immunostimulatory** Nucleic Acids

DETD . . . described (Uhlmann, E. And Peyman, A. (1990) Chem. Rev. 90:544; Goodchild, J. (1990) Bioconjugate Chem. 1:165). 2'-O-methyl nucleic acids with **CpG** motifs also cause immune activation, as do ethoxy-modified **CpG** nucleic acids. In fact, no backbone modifications have been found that completely abolish the **CpG** effect, although it is greatly reduced by replacing the C with a 5-methyl C.

DETD Therapeutic Uses of **Immunostimulatory** Nucleic Acid Molecules

DETD Based on their **immunostimulatory** properties, nucleic acid molecules containing at least one unmethylated **CpG** dinucleotide can be administered to a subject in vivo to treat an "immune system deficiency". Alternatively, nucleic acid molecules containing at least one unmethylated **CpG** dinucleotide can be contacted with lymphocytes (e.g. B cells, monocytic cells or NK cells) obtained from a subject having an. . .

DETD As reported herein, in response to unmethylated **CpG** containing nucleic acid molecules, an increased number of spleen cells secrete IL-6, IL-12, IFNγ, IFN-α, IFN-β, IL-1, IL-3, IL-10, TNF-α,. .

.

DETD **Immunostimulatory** nucleic acid molecules can also be administered to a subject in conjunction with a vaccine to boost a subject's immune system and thereby effect a better response from the vaccine. Preferably the **immunostimulatory** nucleic acid molecule is administered slightly before or at the same time as the vaccine. A conventional adjuvant may optionally. . .

DETD . . . antigen encoded by the vaccine determines the specificity of the immune response. Second, if the backbone of the plasmid contains **CpG** motifs, its functions as an adjuvant for the vaccine. Thus, **CpG** DNA acts as an effective "danger signal" and causes the immune system to respond vigorously to new antigens in the area. This mode of action presumably results primarily from the stimulatory local effects of **CpG** DNA on dendritic cells and other "professional" antigen presenting cells, as well as from the co-stimulatory effects on B cells.

DETD **Immunostimulatory** oligonucleotides and unmethylated **CpG** containing vaccines, which directly activate lymphocytes and co-stimulate an antigen-specific response, are fundamentally different from conventional adjuvants (e.g aluminum precipates),. . .

DETD In addition, an **immunostimulatory** oligonucleotide can be administered prior to along with or after administration of a chemotherapy or immunotherapy to increase the responsiveness. . . chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF. **CpG** nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Induction of NK activity and. . .

DETD Another use of the described **immunostimulatory** nucleic acid molecules is in desensitization therapy for allergies, which are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated **CpG** nucleic acids are predominantly of a class called "Th1" which is most marked; by a cellular immune response and is. . . are mediated by Th2 type immune responses and autoimmune diseases by Th1 immune response. Based on the ability of the **immunostimulatory** nucleic acid molecules to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of an **immunostimulatory** nucleic acid (or a vector containing a nucleic acid) alone or in conjunction with an allergen can be administered to. . .

DETD Nucleic acids containing unmethylated **CpG** motifs may also have significant therapeutic utility in the treatment of asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated. . .

DETD As described in detail in the following Example 12, oligonucleotides containing an unmethylated **CpG** motif (I, e,. **TCCATGACGTTCCTGACGTT**; SEQ ID NO. 10) but not a control oligonucleotide (TCCATGAGCTTCCTGAGTCT; SEQ ID NO. 8) prevented the development of an inflammatory. . .

DETD For use in therapy, an effective amount of an appropriate **immunostimulatory** nucleic acid molecule alone or formulated as a delivery complex can be administered to a subject by any mode allowing. . .

DETD . . . to realize a desired biologic effect. For example, an effective

amount of a nucleic acid containing at least one unmethylated **CpG** for treating an immune system deficiency could be that amount necessary to eliminate a tumor, cancer, or bacterial, viral or. . . such factors as the disease or condition being treated, the particular nucleic acid being administered (e.g the number of unmethylated **CpG** motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or. . .

DETD . . . Table 1). In some experiments, culture supernatants were assayed for IgM by ELISA, and showed similar increased in response to **CpG**-ODN.

DETD . . . C57BL/6 spleen cells were cultured in two ml RPMI (supplemented as described for Example 1) with or without 40 μM **CpG** or non-**CpG** ODN for forty-eight hours. Cells were washed, and then used as effector cells in a short term ^{51}Cr release assay. . .

DETD B cells were cultured with phosphorothioate ODN with the sequence of control ODN 1a or the **CpG** ODN 1d and 3Db and then either pulsed after 20 hr with ^{3H} uridine or after 44 hr with ^{3H}. . .

DETD . . . for 1 hr. At 37° C. in the presence or absence of LPS or the control ODN 1a or the **CpG** ODN 1d and 3Db before addition of anti-IgM (1 μ/ml). Cells were cultured for a further 20 hr. Before a. . .

DETD DBA/2 female mice (2 mos. old) were injected IP with 500 g **CpG** or control phosphorothioate ODN. At various time points after injection, the mice were bled. Two mice were studied for each. . .

DETD . . . 1(2U/ug of DNA) at 37° C. for 2 hr in 1×SSC with 5 mM MgC12. To methylate the cytosine in **CpG** dinucleotide in E. coli DNA, E. coli DNA was treated with **CpG** methylase (M. SssI; 2U/μg of DNA) in NEBuffer 2 supplemented with 160 μM S-adenosyl methionine and incubated overnight at 37°. . .

DETD . . . humidifier incubator maintained in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 1.5 mM L-glutamine, 50 μ/ml), **CpG** or non-**CpG** phosphodiester ODN (O--ODN) (20 μM), phosphorothioate ODN (S--ODN) (0.5 μM), or E. coli or calf thymus DNA (50 μg/ml) at. . . IgM production). Concentrations of stimulants were chosen based on preliminary studies with titrations. In some cases, cells were treated with **CpG** O--ODN along with various concentrations (1-10 μg/ml) of neutralizing rat IgGl antibody against murine IL-6 (hybridoma MP5-20F3) or control rat. . .

DETD . . . injected intravenously (iv) with PBS, calf thymus DNA (200 μg/100 μl PBS/mouse), E. coli DNA (200 μg/100 μl PBS/mouse), or **CpG** or non-**CpG** S--ODN (200 μg/100 μl PBS/mouse). Mice (two/group) were bled by retroorbital puncture and sacrificed by cervical dislocation at various time. . .

DETD Cell Proliferation assay. DBA/2 mice spleen B cells (5×10^4 cells/100 μl/well) were treated with media, **CpG** or non-**CpG** S--ODN (0.5 μM) or O--ODN (20 μM) for 24 hr at 37° C. Cells were pulsed for the last four. . .

DETD . . . a receptor-dependent mechanism. J. Clin. Invest. 93:944) at 250 mV and 960 μF. Cells were stimulated with various concentrations of **CpG** or non-**CpG** ODN after electroporation. Chloramphenicol acetyltransferase (CAT) activity was measured by a solution assay (Seed, B. and J. Y. Sheen (1988). . .

DETD Oligodeoxynucleotide Modifications Determine the Magnitude of B Cell Stimulation by **CpG** Motifs

DETD . . . central linkages are phosphodiester, but the two 5' and five 3' linkages are methylphosphonate modified. The ODN sequences studied (with **CpG** dinucleotides indicated by underlining) include:

DETD These sequences are representative of literally hundreds of **CpG** and non-**CpG** ODN that have been tested in the course of these studies.

DETD . . . (1990) J. Immunol 145:1039; and Ballas, Z. K. and W. Rasmussen (1193) J. Immunol, 150:17), with medium alone or with **CpG** or non-**CpG** ODN at the indicated concentrations, or with E. coli or calf thymus (50 μg/ml) at 37° C. for 24 hr.. . .

DETD . . . mice were then treated with oligonucleotides (30 μg in 200 μl saline by i.p. injection), which either contained an unmethylated **CpG** motif (i e., **TCCATGACGTTCCTGACGTT**; SEQ ID NO.10) or did the (i.e., control, TCCATGAGCTTCCTGAGTCT; SEQ ID NO. 8). Soluble SeEA (10 μg in 25 μl. . .

DETD . . . inflammatory cells are present in the lungs. However, when the mice are initially given a nucleic acid containing an unmethylated **CpG** motif along with the eggs, the inflammatory cells in the lung are not increased by subsequent inhalation of the egg. . .

DETD . . . inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a **CpG** oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes. . .

DETD FIG. 14 shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can actually redirect the cytokine response of the lung to production of II-12, indicating the Th1 type of immune. .

DETD FIG. 15 shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune. .

DETD **CpG** Oligonucleotides Induce Human PBMC to Secrete Cytokines

DETD . . . by standard centrifugation over Ficoll hypaque. Cells (5×10^5/ml) were cultured in 10% autologous serum in 95 well microtiter plates with **CpG** or control oligodeoxynucleotides (24 μg/ml for phosphodiester oligonucleotides; 6 g/ml for nuclease resistant phosphorothioate oligonucleotides) for 4 hr in the. . .

1. A method for increasing the responsiveness of a cancer cell to a cancer therapy using an **immunostimulatory** nucleic acid as compared to the absence of the **immunostimulatory** nucleic acid, comprising: administering to a subject having a cancer an effective amount for increasing the responsiveness of a cancer cell to a cancer therapy of an **immunostimulatory** nucleic acid, comprising: 5'$X_{1X2CGX3X43}$' wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides, and wherein the sequence is not. . .

. . . claim 1, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

25. A method for enhancing recovery of bone marrow using an **immunostimulatory** nucleic acid as compared to the absence of the **immunostimulatory** nucleic acid in a subject undergoing or having undergone cancer therapy, comprising: administering to a subject undergoing or having undergone cancer therapy which damages the bone marrow an effective amount for enhancing the recovery of bone marrow of an **immunostimulatory** nucleic acid, comprising: 5'$X_{1X2CGX3X43}$' wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides.

. . . claim 25, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

30. In a method for stimulating an immune response in a subject having a cancer, the method of the type involving antigen dependent cellular cytotoxicity (ADCC), the improvement comprising: administering to the subject an **immunostimulatory** nucleic acid, comprising: 5'$X_{1X2CGX3X43}$' wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides.

. . . claim 32, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

36. A method for treating or preventing cancer, comprising: administering to a subject having a cancer an effective amount for treating or preventing cancer of an **immunostimulatory** nucleic acid, comprising: 5'$X_{1X2CGX3X43}$' wherein C is unmethylated, wherein X_{1X2} and X_{3X4} are nucleotides, and wherein the sequence is not. . .

. . . claim 36, wherein X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

L17 ANSWER 3 OF 8 USPATFULL on STN

2002:194879 **Immunostimulatory** nucleic acid molecules for activating dendritic cells.

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US 6429199 B1 20020806
APPLICATION: US 1998-191170 19981113 (9)
DOCUMENT TYPE: Utility; GRANTED.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A method for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate a dendritic cell, wherein the method is performed ex vivo.

2. A method for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic cell, wherein the dendritic cell is an isolated dendritic cell.

3. The method of claim 1, wherein the isolated nucleic acid has a formula: 5'$N_{1X1CGX2N23}$' wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymine; X_2 is cytosine, adenine, or thymine; N is any nucleotide and N_1+N_2 is from about 0-25 nucleotides.

4. The method of claim 2, wherein the method is performed ex vivo.

5. The method of claim 4, further comprising contacting the dendritic cell with an antigen prior to the isolated nucleic acid.

6. A method for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic cell, wherein at least one nucleotide of the isolated nucleic acid has a phosphate backbone modification where in the method is peformed ex vivo.

7. The method of claim 6, wherein the phosphate backbone modification is a phosphorothioate or phosphorodithioate modification.

8. The method of claim 7, wherein the phosphate backbone modification occures at the 5' end of the nucleic acid.

9. The method of claim 8, wherein the nucleic acid backbone includes the phosphate backbone modification at the 5' intemucleotide linkages.

10. The method of claim 7, wherein the nucleic acid backbone includes the phosphate backbone modification at the 3' intemucleotide linkages.

11. The method of claim 10, wherein the phosphate backbone modification occurs at the last five internucleotide linkages of the 3' end of the nucleic acid.

12. The method of claim 1, wherein the isolated nucleic acid has a formula: 5'$N_{1X1X2CGX3X4N23}$' wherein at least one nucleotide separates consecutive CpGs; X_{1X2} is selected from the group consisting of TpT, CpT, TpC, and ApT; X_{3X4} is selected from the group consisting of GpT, GpA, ApA and ApT; N is any nucleotide and N_1+N_2 is from about 0-25 nucleotides.

13. A method for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide in an amount effective to activate the dendritic cell, wherein the isolated nucleic acid is selected from the group consisting of SEQ ID Nos. 84 and 85.

14. A method for cancer immunotherapy, comprising: administering an activated dendritic cell that expresses a specific cancer antigen to a subject having a cancer including the cancer antigen, wherein the activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic cell.

15. A method for treating an infectious disease, comprising: administeri ng an activated dendritic cell that expresses a specific microbial antigen to a subject having an infection with a microorganism including the microbial antigen, wherein the activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic cell.

16. A method for treating an allergy, comprising: administering an activated dendritic cell that expresses a specific allergy causing antigen to a subject having an allergic reaction to the allergy causing antigen, wherein the activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic is from about 8-80 bases in length in an amount effective to activate the dendritic cell.

17. A method for generating a high yield of dendritic cells, comprising: administering an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective for activating dendritic cells to a subject; allowing the isolated nucleic acid to activate dendritic cells of the subject; and isolating dendritic cells from the subject.

18. A method for causing maturation of a dendritic cell, comprising contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to cause maturation of the dendritic cell.

19. A method for activating a dendritic cell, comprising: contacting a dendritic cell with an effective amount to activate a dendritic cell of an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide and an antigen.

20. The method of claim 19, wherein the dendritic cell is contacted with the antigen within 48 hours of contacting the dendritic cell with the isolated nucleic acid.

21. The method of claim 19, wherein the dendritic cell is contacted with the antigen within 24 hours of contacting the dendritic cell with the isolated nucleic acid.

TI **Immunostimulatory** nucleic acid molecules for activating dendritic cells
AI **US 1998-191170 19981113 (9)**
AB . . . activating dendritic cells. In particular, the invention relates to oligonucleotides which have a specific sequence including at least one unmethylated **CpG** dinucleotide which are useful for activating dendritic cells. The methods are useful for in vitro, ex-vivo, and in vivo methods. . .
SUMM . . . activating dendritic cells. In particular, the invention relates to oligonucleotides which have a specific sequence including at least one unmethylated **CpG** dinucleotide which are useful for activating dendritic cells.
SUMM . . . the vertebrate immune system has the ability to recognize the presence of bacterial DNA based on the recognition of so-called **CpG**-motifs, unmethylated cytidine-guanosine dinucleotides within specific patterns of flanking bases. According to these disclosures **CpG** functions as an adjuvant and is as potent at inducing B-cell and T-cell responses as the complete Freund's adjuvant, but is preferable since **CpG** induces a higher Th1 response and is less toxic. Alum, the adjuvant which is used routinely in human vaccination, induces the less favorable Th2 response. Compared to alum, **CpG** is a more effective adjuvant. The combination of **CpG** and alum was found to produce a synergistic adjuvant effect.
SUMM **CpG** oligonucleotides also show adjuvant effects towards various immune cells. For instance, **CpG** enhances the efficacy of monoclonal antibody therapy, thus functioning as an effective immune adjuvant for antigen immunization in a B cell lymphoma model. Cytotoxic T cell responses to protein antigen also are induced by **CpG**. Furthermore, the presence of **immunostimulatory** DNA sequences in plasmids was found to be necessary for effective intradermal gene immunization.
SUMM It was discovered according to an aspect of the invention that the adjuvant activity of **CpG** is based on the direct activation of dendritic cells by **CpG**. Potent **immunostimulatory CpG** oligonucleotides and control oligonucleotides were found to cause dramatic changes in dendritic cells isolated from peripheral blood by immunomagnetic cell sorting. **CpG** oligonucleotides provided excellent Dendritic cell survival, differentiation, activation and maturation, and were superior to the combination of GM-CSF and LPS. In fact, the combination of **CpG** and GM-CSF produced unexpected synergistic effects on the activation of dendritic cells. The invention thus encompasses both **CpG** oligonucleotides and the combination of **CpG** oligonucleotides and cytokines such as GM-CSF as well as in vitro, ex vivo, and in vivo methods of activating dendritic. . .
SUMM . . . The method includes the steps of contacting a dendritic cell

with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate a dendritic. . .

SUMM The isolated nucleic acid is one which contains at least one unmethylated **CpG** dinucleotide and which is from about 8-80 bases in length. In one embodiment the unmethylated **CpG** dinucleotide has a formula:

SUMM . . . cytosine, adenine, or thymine; N is any nucleotide and N_1+N_2 is from about 0-25 nucleotides. In another embodiment the unmethylated **CpG** dinucleotide has a formula:

SUMM . . . dendritic cell to an antigen; contacting the isolated dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the isolated nucleic acid is from about 8-80 bases in length; and allowing the isolated dendritic cell to. . .

SUMM The isolated nucleic acid is one which contains at least one unmethylated **CpG** dinucleotide and which is from about 8-80 bases in length. In one embodiment the unmethylated **CpG** dinucleotide has a formula:

SUMM . . . cytosine, adenine, or thymine; N is any nucleotide and N_1+N_2 is from about 0-25 nucleotides. In another embodiment the unmethylated **CpG** dinucleotide has a formula:

SUMM . . . including an effective amount for synergistically activating a dendritic cell of an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length; and an effective amount for synergistically activating a. . .

SUMM . . . assay includes the following steps: contacting an immature dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length; exposing the dendritic cell to a putative drug;. . .

SUMM . . . yield of dendritic cells. The method includes the following steps administering an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective for activating dendritic cells. . .

SUMM . . . The method includes the following steps: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to produce a CD40. . .

SUMM . . . The method includes the step of contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to cause maturation of. . .

DRWD FIG. 1 shows FACS chart depicting **CpG** oligonucleotide promoted survival of dendritic precursor cells. Freshly isolated dendritic precursor cells were incubated for 2 days in the presence. . . of either oligonucleotides or GMCSF (800 U/ml). Flow cytometric analysis of morphology (forward scatter, FSC; sideward scatter, SSC) showed that **CpG** oligonucleotides (2006: **CpG** phosphorothioate oligonucleotide, 1×2 μg/ml, 2080 **CpG** phosphodiester oligonucleotide, 3×30 μg/ml) promote survival of dendritic precursor cells, while the non **CpG** controls (2117: 2006 with methylated **CpG**; 2078: identical to 2080 but GpCs instead of CpGs) showed no positive effect on cell survival compared to the sample. . .

DRWD FIG. 2 is a graph showing that the combination of **CpG** and GMCSF enhances viability of dendritic cells. Dendritic precursor cells were isolated from peripheral blood and incubated for 48 hours with GMCSF (800 U/ml) and oligonucleotides (2006: **CpG** phosphorothioate; 2117: CpGs in 2006 methylated; 2 μg/ml) as indicated. Viability was examined by flow cytometry. Data represent the mean. . .

DRWD . . . as indicated and examined by flow cytometry (sideward scatter, SSC). Viable cells (2500 per sample) were counted. Phosphodiester oligonucleotides (2080: **CpG**; 2078: non-**CpG**) were added at 0 hours, 12 hours and 24 hours (30 μg/ml each time point).

DRWD FIG. 4 shows FACS charts demonstrating that ICAM-1 and MHC II expression of dendritic cells in response to GMCSF and **CpG**. Dendritic precursor cells were incubated for 48 hours in the presence of GMCSF (800 U/ml) and 2006 (**CpG** phosphorothioate; 6 μg/ml). Expression of ICAM-1 (CD54) and MHC II was examined by flow cytometry (2500 viable cells are counted. . .

DRWD FIG. 5 is graphs depicting induction of co-stimulatory molecule expression on dendritic cells by **CpG**. Dendritic precursor cells were incubated for 48 hours in the presence of GMCSF (800 U/ml) and oligonucleotides (2006: **CpG** phosphorothioate, 6 μg/ml) as indicated. Expression of CD54 (ICAM-1) (panel A), CD86 (B7-2) (panel B) and CD40 (panel C) was. . .

DRWD FIG. 6 is graphs depicting the enhancement of CD40 expression on dendritic cells is **CpG** specific and not induced by LPS. Dendritic

precursor cells are cultured for 48 hours in the presence of GMCSF (800 U/ml), LPS (10 ng/ml) and oligonucleotides (2006, **CpG** phosphorothioate, 6 µg/ml: 2117, methylated 2006; 2080 **CpG** phosphodiester, 30 µg/ml at 0 hours, 12 hours and 24 hours; 2078 GpC version of 2080). CD40 expression is examined. . . mean fluorescence intensity). Panel A and panel B show the results of two separate sets of experiments. Panel A shows **CpG** specificity (methylated control oligonucleotide) for the synergy of **CpG** and GMCSF for induction of CD40 expression. Panel B shows that **CpG** is equally effective in enhancing CD40 expression as GMCSF, and that this effect is **CpG** specific (GpC control oligonucleotide). Panel A and B represent the mean of two independent experiments each.

DRWD FIG. 7 is graphs depicting the induction of CD54 and CD86 expression on dendritic cells is **CpG** specific and not induced by LPS. Dendritic precursor cells are cultured for 48 hours in the presence of GMCSF (800 U/ml), LPS (10 ng/ml) and oligonucleotides (2006, **CpG** phosphorothioate, 2 µg/ml: 2117, methylated 2006). CD54 (panel A) and CD86 (panel B) expression is examined by flow cytometry (MFI,. . .

DRWD FIG. 8 shows FACS charts demonstrating that CD86 expression on monocyte-derived Dendritic cells is induced by LPS but not by **CpG**. CD14-positive monocytes were prepared from PBMC by immunomagnetic separation and incubated in the presence of GMCSF (800 U/ml) and IL-4. . . added. CD 86 expression is measured by flow cytometry (numbers represent mean fluorescence intensity). In this series of experiments, the non-**CpG** phosphorothioate control oligonucleotide 2041 (5'-CTG GTC TTT CTG GTT TTT TTC TGG-3') (SEQ ID NO: 93) was used. The results are representative for 8 independent experiments, in which **CpG** did not stimulate monocyte-derived dendritic cells.

DRWD FIG. 9 shows FACS charts demonstrating that **CpG** induces maturation (CD83 expression) of dendritic cells. After 48 hours incubation with GMCSF (800 U/ml), LPS (10 ng/ml) and oligonucleotides (2006: **CpG** phosphorothioate; 2117 methylated 2006; 2 µg/ml), CD83 and CD86 expression on dendritic cells is determined in flow cytometry. Values (%). . .

DRWD FIG. 10 are electron micrographs depicting **CpG** induction of morphologic changes in dendritic cells. Dendritic cells were incubated for 2 days in the presence of GMCSF (800. . .

DRWD FIG. 11 are electron micrographs depicting Ultrastructural differences due to **CpG** Dendritic cells were incubated for 2 days in the presence of GMCSF (800 U/ml) and 2006 (2 µg/ml) (panel A) or with GMCSF (800 U/ml) (panel B) and transmission electron microscopy was performed. In the presence of **CpG** (panel A) multilamellar bodies (>) and multivesicular structures can be seen.

DRWD FIG. 12 are electron micrographs depicting High magnification of **CpG**-characteristic ultrastructural differences. Dendritic cells incubated with GMCSF (800 U/ml) and 2006 (2 µg/ml) were examined by transmission electron microscopy. Arrows. . .

DETD . . . receptors which detect microbial molecules like LPS in their local environment. It has been discovered according to the invention that **CpG** has the unique capability to promote cell survival, differentiation, activation and maturation of dendritic cells. In fact dendritic precursor cells. . . a two day incubation with GM-CSF. Without GM-CSF these cells undergo apoptosis. It was discovered according to the invention that **CpG** was superior to GM-CSF in promoting survival and differentiation of dendritic cells (MHC II expression, cell size, granularity). As shown in the Examples below, the **CpG** phosphorothioate oligonucleotide 2006 (2 µg/ml) induced the expression of ICAM-1 (CD54) by 3.6-fold (p=0.02; n=5), the co-stimulatory molecule B7-2 (CD86). . . either GM-CSF alone or GM-CSF combined with LPS. Electron microscopy revealed major ultrastructural changes of dendritic cells in response to **CpG**, indicating that these cells were differentiated. Additionally **CpG** was found to induce maturation of dendritic cells. **CpG** oligonucleotide 2006 was superior to GM-CSF and LPS at inducing maturation marker CD83. A synergistic maturation effect was observed when **CpG** oligonucleotide 2006 and GM-CSF were used together.

DETD All effects of **CpG** on dendritic cells were **CpG**-specific as shown by control oligonucleotides with methylated **CpG** motifs and oligonucleotides containing GpC instead of **CpG**. Thus, the addition of a **CpG** oligonucleotide is sufficient for improving survival, differentiation, activation and maturation of human dendritic cells. Since dendritic cells form the link between the innate and the acquired immune system the ability to activate dendritic cells with **CpG** supports the use of **CpG**-based strategies for immunotherapy against disorders such as cancer and allergic or infectious diseases.

DETD . . . and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor, N.Y., current edition). It is shown according to the invention that **CpG** functions as an adjuvant by activating dendritic cells. **CpG** is a particularly useful adjuvant in humans because of its low toxicity. Many

potent adjuvants in mice or other animals,. . . like the Freunds complete adjuvant, cannot be used in humans due to toxicity. Additionally, as demonstrated in the examples below, **CpG** activates and matures human primary blood dendritic cells where other known adjuvants such as LPS fail to do so. Furthermore, **CpG** is known to induce a Th1 immune response which is believed to be superior to the immune response induced by. . .

DETD Thus the use of **CpG** allows the generation of mature dendritic cells from peripheral blood within two days in a well defined system. The application of **CpG** for this purpose is superior to GM-CSF, which is currently used for this purpose. **CpG** oligonucleotides have a longer half life, are less expensive, and show a greater magnitude of immune effects. The combination of **CpG** and GM-CSF shows synergistic activity for the induction of co-stimulatory molecules (CD86, CD40).

DETD . . . activating dendritic cells for in vitro, ex vivo and in vivo purposes. It was demonstrated according to the invention that **CpG** oligodeoxyribonucleotides are potent activators of dendritic cells. Dendritic cells are believed to be essential for the initiation of primary immune responses in immune cells in vivo. It was discovered, according to the invention, that **CpG** oligodeoxyribonucleotide was capable of activating dendritic cells to initiate primary immune responses in T cells, similar to an adjuvant. It was also discovered the **CpG** ODN induces the production of large amounts of IL-12 in dendritic cells, indicating its propensity to augment the development of Th1 immune responses in vivo. The findings that **CpG** oligonucleotides were sufficient for survival, differentiation, activation, and maturation of human dendritic cells demonstrate the potent adjuvant activity of **CpG** and provide the basis for the use of **CpG** oligodeoxyribonucleotides as immunotherapeutics in the treatment of disorders such as cancer, infectious diseases, and allergy. In one aspect, the invention. . . for activating a dendritic cell by contacting the dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide, wherein the nucleic acid is from about 8-80 bases in length.

DETD . . . to immunization. This is accomplished by contacting immature dendritic cells with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide to cause the dendritic cell to become activated and to mature. The activated dendritic cell is then incubated with. . .

DETD One specific use for the **CpG** nucleic acids of the invention is to activate dendritic cells for the purpose of enhancing a specific immune response against. . . active against a specific cancer antigen, the dendritic cells may be exposed to the cancer antigen in addition to the **CpG**. In other cases the dendritic cell may have already been exposed to antigen but may not be expressing the antigen. . . the invention may be performed by routine ex vivo manipulation steps known in the art, but with the use of **CpG** as the activator.

DETD The dendritic cells may also be contacted with **CpG** using in vivo methods. In order to accomplish this, **CpG** is administered directly to a subject in need of immunotherapy. The **CpG** may be administered in combination with an antigen or may be administered alone. In some embodiments, it is preferred that the **CpG** be administered in the local region of the tumor.

DETD The isolated dendritic cell is contacted with **CpG** and exposed to an antigen. Although either step may be performed first or the steps may be performed simultaneously, in one preferred embodiment the antigen is exposed to the immature dendritic cell before the cell is contacted with the **CpG**. It is believed that the antigen is taken up by the dendritic cell and then when the dendritic cell is contacted with the **CpG**, that the dendritic cell is activated to process and present the antigen. Preferably, the antigen is exposed to the cell within 48 hours of adding **CpG**. In a more preferred embodiment, the dendritic cell is exposed to the antigen within 24 hours of the **CpG**.

DETD . . . An infectious disease, as used herein, is a disease arising from the presence of a foreign microorganism in the body. **CpG** is used to stimulate an antigen specific dendritic cell which can activate a T cell response against an antigen of. . .

DETD . . . active disorders, the methods and products of the invention can be used as a prophylactic vaccine. In this case, the **CpG** nucleic acid sequence is administered in vivo, preferably in the presence of an antigen or dendritic cells are prepared ex. . .

DETD The **CpG** oligonucleotides of the invention are **immunostimulatory** molecules. An "**immunostimulatory** nucleic acid molecule" refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (i.e. "**CpG** DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates (e.g. has a mitogenic effect on, or induces or increases cytokine expression by) a dendritic cell. An **immunostimulatory** nucleic acid molecule can be double-stranded or single-stranded. Generally,

double-stranded molecules are more stable in vivo, while single-stranded molecules have. . .

DETD In one preferred embodiment the invention provides an isolated **immunostimulatory** nucleic acid sequence containing a **CpG** motif represented by the formula:

DETD In another embodiment the invention provides an isolated **immunostimulatory** nucleic acid sequence containing a **CpG** motif represented by the formula:

DETD Preferably the **immunostimulatory** nucleic acid sequences of the invention include X_{1X2} selected from the group consisting of GpT, GpG, GpA and ApA and X_{3X4} is selected from the group consisting of TpT, CpT and GpT. For facilitating uptake into cells, **CpG** containing **immunostimulatory** nucleic acid molecules are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size (even many kb long) are **immunostimulatory** if sufficient **immunostimulatory** motifs are present, since larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not include. . . or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus mitogenic **CpG** motif is not a palindrome. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, where the oligonucleotide incorporates a phosphate backbone. . .

DETD Preferably the **immunostimulatory CpG** DNA is in the range of between 8 to 30 bases in size when it is an oligonucleotide. Alternatively, **CpG** dinucleotides can be produced on a large scale in plasmids, which after being administered to a subject are degraded into oligonucleotides. Preferred **immunostimulatory** nucleic acid molecules (e.g. for use in increasing the effectiveness of a vaccine or to treat an immune system deficiency. . .

DETD . . . in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated **CpG** containing nucleic acid molecules that are tens to hundreds of kbs long are relatively resistant to in vivo degradation. For shorter **immunostimulatory** nucleic acid molecules, secondary structure can stabilize and increase their effect. For example, if the 3' end of a nucleic. . .

DETD . . . modified backbone. It was shown according to the invention that modification of the oligonucleotide backbone provided enhanced activity of the **CpG** molecules of the invention when administered in vivo. **CpG** constructs, including at least two phosphorothioate linkages at the 5' end of the oligodeoxyribonucleotide and multiple phosphorothioate linkages at the. . .

DETD Both phosphorothioate and phosphodiester oligonucleotides containing **CpG** motifs were active in dendritic cells. However, based on the concentration needed to induce **CpG** specific effects, the nuclease resistant phosphorothioate backbone **CpG** oligonucleotides were more potent (2 μg/ml for the phosphorothioate vs. a total of 90 μg/ml for phosphodiester). In the concentration used in this study, phosphorothioate oligonucleotides without **CpG** motifs showed no background stimulatory activity such as that described earlier for high phosphorothioate oligonucleotide concentrations.

DETD . . . TCGTCGCTGTTGTCGTTTCTT (SEQ ID NO: 77), TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 84), TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO: 85) TGTCGTTGTCGTTGTCGTT (SEQ ID NO: 90), **TCCATGACGTTCCTGACGTT** (SEQ ID NO: 97), GTCG(T/C)T and TGTCG(T/C)T.

DETD The stimulation index of a particular **immunostimulatory CpG** DNA can be tested in various immune cell assays. Preferably, the stimulation index of the **immunostimulatory** CgG DNA with regard to B cell proliferation is at least about 5, preferably at least about 10, more preferably. . . treat an immune system deficiency by stimulating a cell-mediated (local) immune response in a subject, it is important that the **immunostimulatory CpG** DNA be capable of effectively inducing cytokine secretion by dendritic cells.

DETD Preferred **immunostimulatory CpG** nucleic acids should effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml. . . IL-6, 200 pg/ml IL-12, depending on the therapeutic indication, as determined by the assays described in the Examples. Other preferred **immunostimulatory CpG** DNAs should effect at least about 10%, more preferably at least about 15% and most preferably at least about 20%. . .

DETD . . . found that the motifs that stimulate murine cells best differ from those that are more effective with human cells. Certain **CpG** oligodeoxynucleotides are poor at activating human cells (oligodeoxyribonucleotide 1707, 1708, which contain the palindrome forming sequences GACGTC and CACGTG, respectively).

DETD The **CpG** oligonucleotides are used to induce survival, activation, maturation, and differentiation of dendritic cells. A dendritic cell has its ordinary meaning. . .

DETD . . . to the invention may be isolated from any source as long as the

cell is capable of being activated by **CpG** to produce an active antigen expressing dendritic cell. Several in vivo sources of immature dendritic cells may be used according. . . marrow dendritic cells and peripheral blood dendritic cells are both excellent sources of immature dendritic cells that are activated by **CpG**. Other sources may easily be determined by those of skill in the art without requiring undue experimentation, by for instance, isolating a primary source of dendritic cells and testing activation by **CpG** in vitro (e.g., using assays described in the Examples section). The invention also encompasses the use of any immature dendritic cells maintained in culture as a cell line as long as the cell is capable of being activated by **CpG**. Such cell types may be routinely identified using standard assays known in the art.

DETD . . . that are known to be activated by cytokines to produce antigen presenting dendritic cells are capable of being activated by **CpG**. For instance, monocyte-derived dendritic cells are not activated by **CpG**. Recently, the method of monocyte-derived dendritic cells has attracted major attention because the incubation of purified CD14-positive monocytes with GM-CSF. . . situation. Although these cells are highly responsive to LPS it was discovered that monocyte-derived Dendritic cells do not respond to **CpG** (see Examples). It was also demonstrated that human monocytes, while highly sensitive to LPS, show a minor and delayed response to **CpG**.

DETD Peripheral blood dendritic cells isolated by immunomagnetic cell sorting, which are activated by **CpG**, represent a more physiologic cell population of dendritic cells than monocyte derived dendritic cells. Immature dendritic cells comprise approximately 1-3%. . . flow cytometry. Freshly isolated dendritic cells cultured in the absence of GM-CSF rapidly undergo apoptosis. Strikingly, in the presence of **CpG** oligonucleotides without addition of GM-CSF, both cell survival and differentiation is markedly improved compared to GM-CSF. In the presence of **CpG**, dendritic cells form cell clusters which when examined by ultrastructural techniques such as electron microscopy revealed characteristic dense multilamellar intracytoplasmic. . . and had only minor cellular processes. In addition to promoting survival and differentiation of dendritic cells, a single addition of **CpG** oligonucleotide led to activation as represented by upregulation of the co-stimulatory molecules ICAM-1 (CD54), B7-2 (CD86) and CD40. The combination of **CpG** oligonucleotide and GM-CSF enhanced the expression of CD86 and CD40 synergistically, proving that activation is not due to **CpG**-induced GM-CSF.

DETD In addition to activating dendritic cells **CpG** was capable of causing maturation of the dendritic cells. Maturation is assessed by the appearance of CD83, a specific marker for mature human dendritic cells. The presence of **CpG** alone for two days was sufficient to cause maturation of a variable percentage of the cells and the combination of GM-CSF and **CpG** was found to act synergistically to cause maturation of an even greater number of cells.

DETD Each of the effects observed by culturing cells in the presence of **CpG**, improved survival, differentiation, activation and maturation of dendritic cells, were **CpG** specific since control oligonucleotides with methylated CpGs and oligonucleotides with GpC instead of CpGs were inactive. Additionally, **CpG** was superior to LPS in inducing both activation and maturation.

DETD . . . dendritic cells plays a key role for the induction of cytotoxic T-cells from naive T-cells. The profound changes observed in **CpG**-stimulated dendritic cells are similar to those seen after activation by CD40 Lanzavecchia A. Licence to kill. Nature 1998; 393: 413-414.. . . signal under physiologic circumstances. In addition to the data presented herein the data presented in the parent application indicate that **CpG** may be substitutes for CD40 ligation on dendritic cells. CD40 and **CpG** perform a number of parallel actions. First, **CpG** and CD40 both activate c-Jun NH2-terminal kinase and p38, but do not activate the extracellular receptor kinase in B cells. Second, CD40 and **CpG** are each sufficient to induce proliferation of B-cells. Finally, both CD40 and **CpG** activate NK cells in an IL-12 dependent manner.

DETD The ability of **CpG** to activate human dendritic cells differs from that of murine dendritic cells. It has also been discovered that **CpG** upregulates MHC class II and co-stimulatory molecules on murine Langerhans cells. In another study similar changes were described for murine. . . bone marrow-derived Dendritic cells. Sparwasser T, et al. Eur J Immunol 1998; 28: 2045-2054. In both studies the efficacy of **CpG** to induce co-stimulatory molecules does not exceed the effects seen for LPS, to which monocytic cells are highly sensitive. Murine monocytes/macrophages are known to secrete high amounts of inflammatory cytokines in response to **CpG**. Since the murine cell preparation may include other myelomonocytic cells in the analysis as well a secondary indirect effect of **CpG** on Dendritic cells in these cell preparations may have contributed to the described activation of Dendritic cells.

DETD It has been shown according to the invention that purified human blood dendritic cells are highly sensitive to **CpG**, while their response to LPS is barely detectable. The LPS concentration used in this study (10 ng/ml) is 10-fold higher. . . In contrast to human macrophages, the low sensitivity of human blood dendritic cells to LPS and the high sensitivity to **CpG** is striking.

DETD Certain Unmethylated **CpG** Containing Nucleic Acids Were Initially Demonstrated to Have B Cell Stimulatory Activity as Shown In Vitro and In Vivo

DETD . . . the other nonstimulatory control oligodeoxyribonucleotide. In comparing these sequences, it was discovered that all of the four stimulatory oligodeoxyribonucleotide contained **CpG** dinucleotides that were in a different sequence context from the nonstimulatory control.

DETD To determine whether the **CpG** motif present in the stimulatory oligodeoxyribonucleotide was responsible for the observed stimulation, over 300 oligodeoxyribonucleotide ranging in length from 5 to 42 bases that contained methylated, unmethylated, or no **CpG** dinucleotides in various sequence contexts were synthesized. These oligodeoxyribonucleotide, including the two original "controls" (ODN 1 and 2) and two. . . then examined for in vitro effects on spleen cells (representative sequences are listed in Table 1). Several oligodeoxyribonucleotides that contained **CpG** dinucleotides induced B cell activation and IgM secretion; the magnitude of this stimulation typically could be increased by adding more **CpG** dinucleotides (Table 1; compare ODN 2 to 2a or 3D to 3Da and 3Db). Stimulation did not appear to result. . .

DETD Mitogenic oligodeoxyribonucleotide sequences uniformly became nonstimulatory if the **CpG** dinucleotide was mutated (Table 1; compare ODN 1 to 1a; 3D to 3Dc; 3M to 3Ma; and 4 to 4a) or if the cytosine of the **CpG** dinucleotide was replaced by 5-methylcytosine (Table 1; ODN 1b, 2b, 3Dd, and 3Mb). Partial methylation of **CpG** motifs caused a partial loss of stimulatory effect (compare 2a to 2c, Table 1). In contrast methylation of other cytosines did not reduce oligodeoxyribonucleotide activity (ODN 1c, 2d, 3De and 3Mc). These data confirmed that a **CpG** motif is the essential element present in oligodeoxyribonucleotide that activate B cells.

DETD In the course of these studies, it became clear that the bases flanking the **CpG** dinucleotide played an important role in determining the murine B cell activation induced by an oligodeoxyribonucleotide. The optimal stimulatory motif was determined to consist of a **CpG** flanked by two 5' purines (preferably a GpA dinucleotide) and two 3' pyrimidines (preferably a TpT or TpC dinucleotide). Mutations of oligodeoxyribonucleotide to bring the **CpG** motif closer to this ideal improved stimulation (e.g. Table 1, compare ODN 2 to 2e; 3M to 3Md) while mutations. . . Table 1, compare ODN 3D to 3Df; 4 to 4b, 4c and 4d). On the other hand, mutations outside the **CpG** motif did not reduce stimulation (e.g. Table 1, compare ODN 1 to 1d; 3D to 3Dg; 3M to 3Me). For. . .

DETD . . . 10 As. Th effect of the G-rich ends is cis; addition of an oligodeoxyribonucleotide with poly G ends but no **CpG** motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated **CpG** were found to be more **immunostimulatory**.

DETD . . . the subsequent fall in stimulation when purified B cells with or without anti-IgM (at a submitogenic dose) were cultured with **CpG** oligodeoxyribonucleotide, proliferation was found to synergistically increase about 10-fold by the two mitogens in combination after 48 hours. The magnitude. . .

DETD Cell cycle analysis was used to determine the proportion of B cells activated by **CpG**-oligodeoxyribonucleotide. **CpG**-oligodeoxyribonucleotide induced cycling in more than 95% of B cells. Splenic B lymphocytes sorted by flow cytometry into CD23-(marginal zone) and. . . as were both resting and activated populations of B cells isolated by fractionation over Percoll gradients. These studies demonstrated that **CpG**-oligodeoxyribonucleotide induce essentially all B cells to enter the cell cycle.

DETD **Immunostimulatory** Nucleic Acid Molecules Block Murine B Cell Apoptosis

DETD . . . are rescued from this growth arrest by certain stimuli such as LPS and by the CD40 ligand. oligodeoxyribonucleotide containing the **CpG** motif were also found to protect WEHI-23 1 from anti-IgM induced growth arrest, indicating that accessory cell populations are not required for the effect. Subsequent work indicates that **CpG** oligodeoxyribonucleotide induce Bcl-x and myc expression, which may account for the protection from apoptosis. Also, **CpG** nucleic acids have been found to block apoptosis in human cells. This inhibition of apoptosis is important, since it should enhance and prolong immune activation by **CpG** DNA.

DETD Method for Making **Immunostimulatory** Nucleic Acids

DETD . . . described (Uhlmann, E. and Peyman, A., 1990, Chem Rev. 90:544;

Goodchild, J., 1990, Bioconjugate Chem. 1:165). 2'-O-methyl nucleic acids with **CpG** motifs also cause immune activation, as do ethoxy-modified **CpG** nucleic acids. In fact, no backbone modifications have been found that completely abolish the **CpG** effect, although it is greatly reduced by replacing the C with a 5-methyl C.

DETD Based on their **immunostimulatory** properties, nucleic acid molecules containing at least one unmethylated **CpG** dinucleotide can be used as described in detail. The nucleic acid molecules may also be used as set forth herein. . .

DETD **Immunostimulatory** nucleic acid molecules can also be administered to a subject in conjunction with a vaccine to boost a subject's immune system and thereby effect a better response from the vaccine. Preferably the **immunostimulatory** nucleic acid molecule is administered slightly before or at the same time as the vaccine. A conventional adjuvant may optionally. . .

DETD . . . antigen encoded by the vaccine determines the specificity of the immune response. Second, if the backbone of the plasmid contains **CpG** motifs, it functions as an adjuvant for the vaccine. Thus, **CpG** DNA acts as an effective "danger signal" and causes the immune system to respond vigorously to new antigens in the area. This mode of action presumably results primarily from the stimulatory local effects of **CpG** DNA on dendritic cells and other "professional" antigen presenting cells, as well as from the co-stimulatory effects on B cells.

DETD **Immunostimulatory** oligonucleotides and unmethylated **CpG** containing vaccines, which directly activate lymphocytes and co-stimulate an antigen-specific response, are fundamentally different from conventional adjuvants (e.g. aluminum precipitates),. . .

DETD In addition, an **immunostimulatory** oligonucleotide can be administered prior to, along with or after administration of a chemotherapy or immunotherapy to increase the responsiveness. . . chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF. **CpG** nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Induction of NK activity and. . .

DETD Another use of the described **immunostimulatory** nucleic acid molecules is in desensitization therapy for allergies, which are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated **CpG** nucleic acids are predominantly of a class called "Th1" which is most marked by a cellular immune response and is. . . are mediated by Th2 type immune responses and autoimmune diseases by Th1 immune response. Based on the ability of the **immunostimulatory** nucleic acid molecules to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of an **immunostimulatory** nucleic acid (or a vector containing a nucleic acid) alone or in conjunction with an allergen can be administered to. . .

DETD Nucleic acids containing unmethylated **CpG** motifs may also have significant therapeutic utility in the treatment of asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated. . .

DETD As described in Co-pending parent patent application U.S. Ser. No. 08/960,774, oligonucleotides containing an unmethylated **CpG** motif (i.e. **TCCATGACGTTCCTGACGTT**; SEQ IN NO: 97), but not a control oligonucleotide (TCCATGAGCTTCCTGAGTCT; SEQ ID NO: 98) prevented the development of an inflammatory. . .

DETD For use in therapy, an effective amount of an appropriate **immunostimulatory** nucleic acid molecule alone or formulated as a delivery complex can be administered to a subject by any mode allowing. . .

DETD . . . to realize a desired biologic effect. For example, an effective amount of a nucleic acid containing at least one unmethylated **CpG** for treating an immune system deficiency could be that amount necessary to eliminate a tumor, cancer, or bacterial, viral or. . . such factors as the disease or condition being treated, the particular nucleic acid being administered (e.g. the number of unmethylated **CpG** motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or. . .

DETD The compositions of the invention, including activated dendritic cells, isolated **CpG** nucleic acid molecules, cytokines, and mixtures thereof are administered in pharmaceutically acceptable compositions. The compositions may be administered by bolus. . .

DETD As reported herein, in response to unmethylated **CpG** containing nucleic acid molecules, an increased number of spleen cells secrete IL-6, IL-12, IFN-γ, IFN-α, IFN-β, IL-1, IL-3, IL-10, TNF-α,. . .

DETD Systemic administration of **CpG** alone in some embodiments is useful for immunotherapy against antigens. Alternative agents like GM-CSF have a shorter half life, although their synergistic effects with **CpG** will likely make this combination useful. On the other hand, some activators

of dendritic cells like LPS or inflammatory cytokines. . . systemic use for this purpose not practical. The present study provides the functional rationale and methods for the use of **CpG** for dendritic cell-based immunotherapeutic strategies against cancer and for its use as an adjuvant in humans.

DETD Systemically administered **CpG** oligonucleotides enhances the availability of immature and mature dendritic cells in the blood and in tissues.

DETD . . . also useful for in vitro screening assays. For instance, immature dendritic cells may be used in vitro to identify other **CpG** specific motifs which are useful for activating or causing maturation of dendritic cells. These motifs may then be used in. . . ex vivo for activating dendritic cells. Additionally, the same type of assay may be used to identify cytokines or other **immunostimulatory** molecules which may have synergistic adjuvant effects when combined with isolated **CpG** nucleic acid sequences of the invention.

DETD . . . maturation. The assay would involve the addition of a putative drug to a immature dendritic cell which is activated by **CpG**. If the putative drug prevents activation, then it may be a compound which is therapeutically capable of inhibiting activation or. . .

DETD . . . CD14, CD16, CD56) (O'Doherty U, et al., "Dendritic cells freshly isolated from human blood express CD4 and mature into typical **immunostimulatory** dendritic cells after culture in monocyte-conditioned medium", J Exp Med, 1993; 178: 1067-1076). Using these characteristics, dendritic cells can be. . .

DETD . . . optimal for immunotherapeutic purposes. We found that monocyte-derived dendritic cells are sensitive to LPS but surprisingly are not activated by **CpG** motifs (FIG. 8). It is believed that the inability of monocyte-derived DC to respond to **CpG** might be due to the unphysiologic methods by which these cells are prepared. Consequently, the effect of **CpG** oligonucleotides on primary peripheral blood DC was examined.

DETD **CpG** Substitutes for GMCSF for DC Survival

DETD . . . their ability to activate human B-cells and NK-cells, we selected particularly potent oligonucleotides as examples of a family of active **CpG**-containing oligonucleotides for the use in the present study. The **CpG** oligonucleotides used were: 2006 (24-mer), 5'-TCG TCG TTT TGT CGT TTT GTC GTT-3' (SEQ ID NO: 84), completely phosphorothioate-modified, and 2080 (20-mer), 5'-TCG TCG TTC CCC CCC CCC CC-3' (SEQ ID NO: 94), un-modified phosphodiester. The non-**CpG** control oligonucleotides used were: 2117 (24-mer), 5'-TQG TQG TTT TGT QGT TTT GTQ GTT-3' (SEQ ID NO: 95), Q=5 methyl. . .

DETD . . . the absence of GMCSF, DC undergo apoptosis during the first two days of cell culture. We examined the effect of **CpG** oligonucleotides on survival of DC in cell culture. Freshly isolated DC were incubated in the presence of GMCSF or oligonucleotides. . . the formation of cell clusters within one day for both the sample with GMCSF alone and the sample with the **CpG** phosphorothioate oligonucleotide 2006. While the size of the clusters was not different between these two samples, the DC incubated with. . . of mature dendritic cells. This difference was distinctive between GMCSF and 2006 samples by using light microscopy. Without GMCSF or **CpG**, no clusters could be found but there was an increasing number of non-viable cells as revealed by trypan blue staining. Viability of DC was quantified by flow cytometry (FIG. 1). Cell survival was dramatically improved in the presence of **CpG** motifs. This effect was found to be **CpG** specific for both phosphorothioate (2006, 2117) and phosphodiester (2080, 2078) oligonucleotides, since both non-**CpG** control oligonucleotides (2117: methylated version of 2006; 2078: CpGs in 2080 inverted to GpCs) showed no improved survival compared to. . .

DETD . . . μg/ml) cell survival was low and comparable to the sample with cells only (10.8+-5.2% and 7.4+-4.2%). These results show that **CpG** can substitute for GMCSF for promoting DC survival, and that the combination of both is favorable over each of them. . .

DETD Increased Size and Granularity of DC Induced by **CpG** is Associated with Enhanced Expression of MHC II

DETD Flow cytometric analysis suggested that differentiation of DC is enhanced by **CpG** and is associated with an increase of cell size (FSC) and granularity (SSC) (FIG. 1). The surface expression of MHC. . . II) and examined by flow cytometry (2500 viable cells counted) (FIG. 3). In the sample with cells only or the non-**CpG** oligonucleotide (2078), a large immature population with low granularity (SSC) and lower MHC II expression was found (FIG. 3 region. . . and high expression of MHC II representing differentiated DC (FIG. 3, region B). The addition of either GMCSF or the **CpG** oligonucleotide 2080 enhanced both granularity and MHC II expression on a per cell basis (FIG. 3 left two panels). The **CpG** oligonucleotide 2080 showed a superior effect compared to GMCSF indicating that **CpG** promotes differentiation of DC in addition to an enhancement of cell survival.

DETD **CpG** Increases Co-stimulatory Molecules on DC

DETD . . . immune response by DC. Functional activation of DC requires by the expression of co-stimulatory molecules. We examined the effect of **CpG** on the expression of the intercellular adhesion molecule-1 (ICAM-1, CD54), and the co-stimulatory surface molecules B7-2 (CD86) and CD40. First,. . . 5, panel C) was quantified in flow cytometry by the mean fluorescence intensity (MFI) of viable DC. In all experiments, **CpG** was superior to GMCSF in enhancing expression of co-stimulatory molecules. Compared to the cells only sample, the **CpG** oligonucleotide 2006 enhanced the expression of CD54 (25.0+-5.7 vs. 7.0+-1.8; p=0.02, n=5), CD86(3.9+-0.8 vs. 1.6+-0.3; p=0.01; n=5) and CD40 (3.5+-1.0. . .

DETD . . . using 2117 (methylated version of 2006) and 2078 (GpC version of 2080). As shown in FIG. 6 for CD40, the non-**CpG** oligonucleotide 2117 showed no synergistic enhancement of CD40 expression when combined with GMCSF (FIG. 6 panel A). The non-**CpG** oligonucleotide 2078 alone did not induce CD40 compared to cells only (FIG. 6 B). Induction of CD86 (FIG. 7 panel A) and CD54 (FIG. 7 panel B) was also found to be **CpG** specific.

DETD . . . the maximal response in terms of cytokine production. Monocyte-derived DC are highly sensitive to LPS but do not respond to **CpG** suggesting major functional differences between monocyte-derived DC and DC isolated from peripheral blood (FIG. 8).

DETD **CpG** Induces Maturation (CD83 expression) of DC

DETD . . . Freshly isolated DC were incubated for 3 days with GMCSF, LPS or oligonucleotides. In the absence of either GMCSF or **CpG**, or with the methylated control oligonucleotide 2117 (2 μg/ml), survival of cells was poor. The remaining viable cells did not. . . 2006 even enhances CD83 expression synergistically (37%) (FIG. 9, left dot plot, upper row). This induction of CD83 expression was **CpG** specific as shown by the control oligonucleotide 2117 in combination with GMCSF (9.7%) (FIG. 9, right dot plot, upper row).. . .

DETD Ultrastructural Changes of DC in Response to **CpG**

DETD We examined DC by electron microscopy to detect ultrastructural differences due to **CpG**. In scanning electron microscopy (FIG. 10), DC cultivated with either GMCSF and **CpG** (FIG. 10 A) or with **CpG** alone (FIG. 10B) displayed a more irregular shape, longer veil processes and sheet-like projections, and more intercellular contacts than cells cultivated with GMCSF alone (FIG. 10C) or in combination with the non-**CpG** control oligonucleotide (FIG. 10D). Transmission electron microscopic imaging revealed striking differences between DC generated with GMCSF combined with **CpG** (FIG. 11A) and GMCSF alone (FIG. 11B). DC generated in the presence of **CpG** showed multilamellar intracytoplasmic bodies of high density (FIG. 11A, FIG. 12, indicated by >), which are absent without **CpG** (FIG. 11B). In addition, **CpG**-generated DC showed prominent multivesicular bodies (FIG. 11A, FIG. 12, indicated by >>), and a less heterochromatin in the nucleus. The. . .

DETD

TABLE 5

ODN Sequence (5'-3') SEQ ID NO.

1754 ACCATGGACGATCTGTTTCCCCTC 61
1758 **TCTCCCAGCGTGCGCCAT** 62
1761 TACCGCGTGCGACCCTCT 63
1776 ACCATGGACGAACTGTTTCCCCTC 64
1777 ACCATGGACGAGCTGTTTCCCCTC 65
1778 ACCATGGACGACCTGTTTCCCCTC 66
1779 ACCATGGACGTACTGTTTCCCCTC 67
1780 ACCATGGACGGTCTGTTTCCCCTC 68
1781. . .

DETD . . . 107

1965 TCCTGTCGTTTTTTGTCGTT 108
1967 TCGTCGCTGTCTGCCCTTCTT 109
1968 TCGTCGCTGTTGTCGTTTCTT 110
2005 TCGTCGTTGTCGTTGTCGTT 111
2006 TCGTCGTTTTGTCGTTTTGTCGTT 112
2014 TGTCGTTGTCGTTGTCGTT 113
2015 TCGTCGTCGTCGTT 114
2016 TGTCGTTGTCGTT 115
1668 TCCATGACGTTCCTGATGCT (SEQ.ID.NO 116)
1758 **TCTCCCAGCGTGCGCCAT** (SEQ.ID.NO 117)

. . . for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate a dendritic. . .

. . . for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic. . .

. . . for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic. . .

. . . for activating a dendritic cell, comprising: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide in an amount effective to activate the dendritic cell, wherein the isolated nucleic acid is selected from the group. . .

. . . activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic. . .

. . . activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to activate the dendritic. . .

. . . activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic is from about 8-80 bases in length in an amount effective to activate the dendritic cell.

. . .

. . . method for generating a high yield of dendritic cells, comprising: administering an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective for activating dendritic cells. . .

. . . maturation of a dendritic cell, comprising contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide wherein the nucleic acid is from about 8-80 bases in length in an amount effective to cause maturation of. . .

. . . cell with an effective amount to activate a dendritic cell of an isolated nucleic acid containing at least one unmethylated **CpG** dinucleotide and an antigen.

L17 ANSWER 4 OF 8 USPATFULL on STN

2002:143951 Use of nucleic acids containing unmethylated **CpG** dinucleotide as an adjuvant.

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US 6406705 B1 20020618

APPLICATION: US **1999-325193 19990603 (9)**

PRIORITY: US 1997-40376P 19970310 (60)

DOCUMENT TYPE: Utility; GRANTED.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A composition of a synergistic combination of adjuvants, comprising: an effective amount for inducing a synergistic adjuvant response of a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant.

2. The composition of claim 1, wherein the non-nucleic acid is an adjuvant that creates a depo effect.

3. The composition of claim 2, wherein the adjuvant that creates a depo effect is selected from the group consisting of alum, emulsion based formulations, mineral oil, non-mineral oil, water-in-oil emulsions, water-in-oil-in-water emulsions, Seppic ISA series of Montanide adjuvants; MF-59; and PROVAX.

4. The composition of claim 1, wherein the non-nucleic acid adjuvant is an immune stimulating adjuvant.

5. The composition of claim 4, wherein the immune stimulating adjuvant is selected from the group consisting of saponins, PCPP polymer; derivatives of lipopolysaccharides, MPL, MDP, t-MDP, OM-174 and Leishmania elongation factor.

6. The composition of claim 1, wherein the non-nucleic acid adjuvant is an adjuvant that creates a depo effect and stimulates the immune system.

7. The composition of claim 6, wherein the adjuvant that creates a depo

effect and stimulates the immune system is selected from the group consisting of ISCOMS, SB-AS2, AS2, SB-AS4, non-ionic block copolymers and SAF.

8. The composition of claim 1, wherein the composition also includes an antigen that is selected from the group consisting of peptides, polypeptides, cells, cell extracts, polysaccharides, polysaccharide conjugates, lipids, glycolipids, carbohydrates, viruses, viral extracts and antigens encoded within nucleic acids.

9. The composition of claim 8, wherein the antigen is derived from an infectious agent selected from the group consisting of a virus, bacterium, fungus and parasite.

10. The composition of claim 8, wherein the antigen is a tumor antigen.

11. The composition of claim 8, wherein the antigen is an allergen.

TI Use of nucleic acids containing unmethylated **CpG** dinucleotide as an adjuvant

AI US 1999-325193 19990603 (9)

AB The present invention relates generally to adjuvants, and in particular to methods and products utilizing a synergistic combination of **immunostimulatory** oligonucleotides having at least one unmethylated **CpG** dinucleotide (**CpG** ODN) and a non-nucleic acid adjuvant. Such combinations of adjuvants may be used with an antigen or alone. The present invention also relates to methods and products utilizing **immunostimulatory** oligonucleotides having at least one unmethylated **CpG** dinucleotide (**CpG** ODN) for induction of cellular immunity in infants.

SUMM . . . to adjuvants, and in particular to methods and products utilizing a synergistic combination of oligonucleotides having at least one unmethylated **CpG** dinucleotide (**CpG** ODN) and a non-nucleic acid adjuvant.

SUMM Bacterial DNA, but not vertebrate DNA, has direct **immunostimulatory** effects on peripheral blood mononuclear cells (PBMC) in vitro (Krieg et al., 1995). This lymphocyte activation is due to unmethylated **CpG** dinucleotides, which are present at the expected frequency in bacterial DNA ({fraction (1/16)}), but are under-represented (**CpG** suppression, {fraction (1/50)} to {fraction (1/60)}) and methylated in vertebrate DNA. Activation may also be triggered by addition of synthetic oligodeoxynucleotides (ODN) that contain an unmethylated **CpG** dinucleotide in a particular sequence context. It appears likely that the rapid immune activation in response to **CpG** DNA may have evolved as one component of the innate immune defense mechanisms that recognize structural patterns specific to microbial. . .

SUMM **CpG** DNA induces proliferation of almost all (>95%) B cells and increases immunoglobulin (Ig) secretion. This B cell activation by **CpG** DNA is T cell independent and antigen non-specific. However, B cell activation by low concentrations of **CpG** DNA has strong synergy with signals delivered through the B cell antigen receptor for both B cell proliferation and Ig. . . al., 1995). This strong synergy between the B cell signaling pathways triggered through the B cell antigen receptor and by **CpG** DNA promotes antigen specific immune responses. In addition to its direct effects on B cells, **CpG** DNA also directly activates monocytes, macrophages, and dendritic cells to secrete a variety of cytokines, including high levels of IL-12. . . activity (Klinman et al., 1996, supra; Cowdery et al., 1996, supra; Yamamoto et al., 1992; Ballas et al., 1996). Overall, **CpG** DNA induces a Th1 like pattern of cytokine production dominated by IL-12 and IFN-γ with little secretion of Th2 cytokines. . .

SUMM . . . and a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant, and wherein the combination of adjuvants is administered in an effective amount. . .

SUMM The **CpG** oligonucleotide and the non-nucleic acid adjuvant may be administered with any or all of the administrations of antigen. For instance. . . antigen. In some embodiments the subject is administered a priming dose of antigen and oligonucleotide containing at least one unmethylated **CpG** dinucleotide before the boost dose. In yet other embodiments the subject is administered a boost dose of antigen and oligonucleotide containing at least one unmethylated **CpG** dinucleotide after the priming dose.

SUMM . . . response a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant, and wherein the combination of adjuvants is administered in an effective amount. . . aspect, the same method is performed but the

subject is an infant and the Th1 response can be induced using **CpG** DNA alone, or **CpG** DNA in combination with a non-nucleic acid adjuvant at the same or different site, at the same or different time.

SUMM . . . of a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant. The composition may also include at least one antigen, which may be. . .

SUMM . . . The method involves the step of administering to an infant an antigen and an oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant in an effective amount for inducing cell mediated immunity or Th1-like responses. . . .

SUMM The **CpG** oligonucleotide may be administered with any or all of the administrations of antigen. For instance the **CpG** oligonucleotide or the combination of adjuvants may be administered with a priming dose of antigen. In another embodiment the **CpG** oligonucleotide or the combination of adjuvants is administered with a boost dose of antigen. In some embodiments the subject is administered a priming dose of antigen and oligonucleotide containing at least one unmethylated **CpG** dinucleotide before the boost dose. In yet other embodiments the subject is administered a boost dose of antigen and oligonucleotide containing at least one unmethylated **CpG** dinucleotide after the priming dose.

SUMM . . . receiving an antigen and at least one non-nucleic acid adjuvant and at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide in order to induce a stronger Th1 immune response than either the adjuvant or oligonucleotide produces alone.

SUMM . . . administering to a subject at least one non-nucleic acid adjuvant and at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide in order to induce a Th1 innate immune response. For longer term protection, these adjuvants may be administered more than once. In another embodiment, **CpG** DNA may be used alone at one or more of the administrations.

SUMM In each of the above described embodiments a **CpG** oligonucleotide is used as an adjuvant. The oligonucleotide in one embodiment contains at least one unmethylated **CpG** dinucleotide having a sequence including at least the following formula:

SUMM In some embodiments X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA. Preferably X_{1X2} are GpA or GpT and X_{3X4} are TpT. In other preferred. . .

SUMM . . . mice immunized with 1 μg recombinant HBsAg protein alone, adsorbed onto alum (25 mg Al^{3+}/mg HBsAg), with 100 μg of **immunostimulatory CpG** ODN 1826, or with both alum and **CpG** ODN. Left panel: Each point represents the group mean (n=10) for titers of anti-HBs (total IgG) as determined in triplicate. . .

SUMM . . . 1 μg recombinant HBsAg protein, with or without alum, and with 0, 0.1, 1, 10, 100 or 500 μg of **CpG** ODN 1826 added. Each point represents the group mean (n=10) for anti-HBs titers (total IgG) as determined by end-point dilution. . .

SUMM . . . phosphorothioate backbone (S) or a chimeric of phosphodiester center regions and phosphorothioate ends (SOS). Most of the ODN contained 1-3 **CpG** motifs but some of the ODN were non-**CpG** controls (1911, 1982, 2041). Each point represents the group mean (n=5) for anti-HBs titers (total IgG) as determined by end-point. . .

SUMM . . . BALB/c mice immunized with 1 μg recombinant HBsAg protein with alum (25 mg Al^{3+}/mg HBs/Ag), with 10 μg of **CpG** ODN 1826, or with both alum and **CpG** ODN. Some animals were boosted with the same or a different formulation after 8 weeks. Each point represents the group. . .

SUMM . . . without adjuvant or with various adjuvants alone or in combination. The adjuvants were: alum (25 mg Al^{3+}/mg HBs/Ag), with **CpG** DNA (10 jig **CpG** ODN 1826), monophosphoryl lipid A (MPL, 50 μg) and Freund's complete adjuvant (mixed 1:1 v/v with HBsAg solution). Each point. . .

SUMM . . . IgG (end-point ELISA titer) produced at 4 weeks in BALB/c mice immunized with 1 μg of HBsAg with or without **CpG** and/or IFA (mineral oil mixed 1:1 v/v) or CFA (complete Freund's adjuvant mixed 1:1 v/v). The numbers above each bar. . .

SUMM . . . amount of total IgG produced at 4 weeks in BALB/c mice immunized with 1 μg of HBsAg with or without **CpG** and/or MPL (monophosphoryl lipid A, 50 μg) or alum. The numbers above each bar indicate the IgG2a:IgG1 ratio, with a. . .

SUMM . . . immunized with 10 μg HBsAg-expressing DNA vaccine (pCMV-S), or with recombinant HBsAg (1 μg) with alum (25 mg Al3+/mg HBsAg), **CpG** ODN 1826 (10 μg) or both alum and **CpG** ODN. Each point represents the proportion of mice responding, the numbers above the bars

show the number of responding over. . .

SUMM . . . pCMV-S), or with 1 μg recombinant HBsAg protein alone, adsorbed onto alum (25 mg Al^{3+}/mg HBsAg), with 100 μg of **immunostimulatory CpG** ODN 1826, or with both alum and **CpG** ODN. Upper panel: Each point represents the group mean of animals that seroconverted (see FIG. 8 for numbers of animals). . .

SUMM . . . 7 days of age) with 1 μg recombinant HBsAg protein with alum (25 mg Al^{3+}/mg HBsAg), with 10 μg of **CpG** ODN 1826, or with both alum and **CpG** ODN. Each point represents the group mean (see FIG. 8 for numbers of animals) for anti-HBs titers (IgG1 and IgG2a. . .

SUMM . . . vaccine (10 μg recombinant HBsAg protein with alum, SmithKline Beecham biologicals, Rixensart, BE) or with Engerix-B plus 500 μg of **CpG** ODN 1968. Each point represents the group mean (n=5) for anti-HBs titers in milli-Intemational units/ml (mIU/ml). A titer of 10. . .

SUMM . . . in millilnternational Units per millilitre (mIU/ml) in orangutans immunized with 10 μg HBsAg with alum (like the HBV commercial vaccine), **CpG** oligonucleotides (**CpG** ODN 2006, 1 mg) or both alum and **CpG** ODN. The numbers above the bars show the number of animals with seroconversion (upper numbers, >1 mIU/ml) or with seroprotection. . .

DETD The invention in one aspect is based on the discovery that formulations containing combinations of **immunostimulatory CpG** oligonucleotides and non-nucleic acid adjuvants synergistically enhance immune responses to a given antigen. Different non-nucleic acid adjuvants used in combination. . .

DETD It has been discovered according to the invention that the combination of **immunostimulatory CpG** oligonucleotides and alum, MPL and other adjuvants results in a synergistic immune response. Compared with the recombinant hepatitis B surface. . . vaccine alone, addition of alum increases the level of antibodies in mice against HBsAg (anti-HBs) about 7-fold whereas addition of **CpG** ODN increases them 32-fold. When **CpG** ODN and alum are used together, a 500-1000 times higher level of anti-HBs was observed, indicating a strong synergistic response.. . . immunization with HBsAg and alum resulted in a strong Th2-type response with almost all IgG being of the IgG1 isotype. **CpG** ODN induced a high proportion of IgG2a, indicative of a Th1-type response, even in the presence of alum. Furthermore, it. . . to the invention that in very young mice (7 day old), immune responses were induced by HBsAg with alum and **CpG** ODN but not with alum or **CpG** ODN alone. The antibodies produced with **CpG** ODN were predominantly of the IgG2a isotype, indicating a strong Th1-type response. This is remarkable considering the strong Th2 bias. . . antibodies. As well, Th1 responses are associated with cytotoxic T lymphocytes (CTL) that can attack and kill virus-infected cells. Indeed, **CpG** ODN, alone or in combination with alum induced good CTL activity in both adult and neonatal mice. These studies demonstrate that the addition of **CpG** ODN to protein or DNA vaccines in combination with other adjuvants is a valid new adjuvant approach to improve efficacy.

DETD . . . and a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant, and wherein the combination of adjuvants is administered in an effective amount. . .

DETD . . . kidney dialysis patients, alcoholics) the rate of non-response can approach 50%. As set forth in the Examples below, alum plus **CpG** ODN gave higher anti-HBs titers than alum alone in a strain of mice which has MHC-restricted hypo-responsiveness to HBsAg, thought to result in a failure to recognize T-helper epitopes. **CpG** ODN also overcame non-response in mice genetically incapable of providing T-help owing to an absence of class II MHC. Similar. . . vaccine with less than 10% achieving seroprotection after 2 doses, but that nearly 100% of animals responded with use of **CpG** oligonucleotides alone or combined with alum. The synergistic response was evident because antibody titers were much higher with **CpG** ODN plus alum than with **CpG** ODN alone or alum alone and were more than additive. These results support the proposition that **CpG** ODN drives the T cell independent activation of B cells. Thus in addition to providing a more effective and easier. . .

DETD . . . specific for the type of cancer to which the subject is at risk of developing and an adjuvant and a **CpG** oligonucleotide the subject may be able to kill of the cancer cells as they develop. If a tumor begins to. . .

DETD Allergies are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated **CpG** oligonucleotides are predominantly of a class called "Th1" which includes IL-12 and IFN-γ. In contrast, Th2 immune response are associated. . .

DETD Based on the ability of the **CpG** oligonucleotides to shift the immune response in a subject from a Th2 (which is associated with production of

IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of a **CpG** oligonucleotide can be administered to a subject to treat or prevent an allergy.

DETD Since Th1 responses are even more potent with **CpG** DNA combined with non-nucleic acid adjuvants, the combination of adjuvants of the present invention will have significant therapeutic utility in. . .

DETD . . . administered a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant. An oligonucleotide containing at least one unmethylated **CpG** dinucleotide is a nucleic acid molecule which contains an unmethylated cytosine-guanine dinucleotide sequence (i.e. "**CpG** DNA" or DNA containing a 5' cytosine followed by 3' guanosine and linked by a phosphate bond) and activates the immune system. The **CpG** oligonucleotides can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have increased immune activity. The **CpG** oligonucleotides or combination of adjuvants can be used with or without antigen.

DETD . . . from existing nucleic acid sources (e.g. genomic or cDNA), but are preferably synthetic (e.g. produced by oligonucleotide synthesis). The entire **CpG** oligonucleotide can be unmethylated or portions may be unmethylated but at least the C of the 5' CG 3' must. . .

DETD In one preferred embodiment the invention provides a **CpG** oligonucleotide represented by at least the formula:

DETD In another embodiment the invention provides an isolated **CpG** oligonucleotide represented by at least the formula:

DETD . . . separates consecutive CpGs; X_{1X2} are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_{3X4} are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA; N is any nucleotide and N_1 and N_2 are nucleic acid sequences composed. . . may have more influence on the biological activity or the kinetics of the biological activity. In another preferred embodiment the **CpG** oligonucleotide has the sequence 5'$TCN_{1TX1X2CGX3X43}$'.

DETD Preferably the **CpG** oligonucleotides of the invention include X_{1X2} selected from the group consisting of GpT, GpG, GpA and ApA and X_{3X4} is selected from the group consisting of TpT, CpT and GpT. For facilitating uptake into cells, **CpG** containing oligonucleotides are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size. . . than 8 nucleotides (even many kb long) are capable of inducing an immune response according to the invention if sufficient **immunostimulatory** motifs are present, since larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not include. . .

DETD Preferably the **CpG** oligonucleotide is in the range of between 8 and 100 and more preferably between 8 and 30 nucleotides in size. Alternatively, **CpG** oligonucleotides can be produced on a large scale in plasmids. These may be administered in plasmid form or alternatively they. . .

DETD The **CpG** oligonucleotide and immunopotentiating cytokine may be directly administered to the subject or may be administered in conjunction with a nucleic. . .

DETD . . . capable of forming the usual Watson-Crick base pairs. In vivo, such sequences may form double-stranded structures. In one embodiment the **CpG** oligonucleotide contains a palindromic sequence. A palindromic sequence used in this context refers to a palindrome in which the **CpG** is part of the palindrome, and preferably is the center of the palindrome. In another embodiment the **CpG** oligonucleotide is free of a palindrome. A **CpG** oligonucleotide that is free of a palindrome is one in which the **CpG** dinucleotide is not part of a palindrome. Such an oligonucleotide may include a palindrome in which the **CpG** is not part of the palindrome.

DETD . . . in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated **CpG** oligonucleotides that are tens to hundreds of kbs long are relatively resistant to in vivo degradation, particularly when in a double-stranded closed-circular form (i.e., a plamid). For shorter **CpG** oligonucleotides, secondary structure can stabilize and increase their effect. For example, if the 3' end of an oligonucleotide has self-complementarity. . .

DETD . . . invention have a modified backbone. It has been demonstrated that modification of the oligonucleotide backbone provides enhanced activity of the **CpG** oligonucleotides when administered in vivo. **CpG** constructs, including at least two phosphorothioate linkages at the 5' end of the oligonucleotide in multiple phosphorothioate linkages at the. . .

DETD Both phosphorothioate and phosphodiester oligonucleotides containing

CpG motifs are active in immune cells. However, based on the concentration needed to induce CpG specific effects, the nuclease resistant phosphorothioate backbone CpG oligonucleotides are more potent (2 μg/ml for the phosphorothioate vs. a total of 90 μg/ml for phosphodiester).

DETD . . . 08/960,774, filed on Oct. 30, 1996 and Oct. 30, 1997 respectively. Exemplary sequences include but are not limited to those **immunostimulatory** sequences shown in Table 1.

DETD The stimulation index of a particular **immunostimulatory** CpG DNA can be tested in various immune cell assays. Preferably, the stimulation index of the CpG oligonucleotide with regard to B cell proliferation is at least about 5, preferably at least about 10, more preferably at. . . on Oct. 30, 1996 and Oct. 30, 1997 respectively. For use in vivo, for example, it is important that the CpG oligonucleotide and adjuvant be capable of effectively inducing activation of Ig expressing B cells. Oligonucleotides which can accomplish this include,. . .

DETD The oligonucleotide containing at least one unmethylated CpG is used in combination with a non-nucleic acid adjuvant and an antigen to activate the immune response. A "non-nucleic acid adjuvant" is any molecule or compound except for the CpG oligonucleotides described herein which can stimulate the humoral and/or cellular immune response. Non-nucleic acid adjuvants include, for instance, adjuvants that. . . adjuvants that create a depo effect and stimulate the immune system. In infants, the oligonucleotide containing at least one unmethylated CpG is used alone or in combination with a non-nucleic acid adjuvant and an antigen to activate a cellular immune response.

DETD When the CpG oligonucleotide containing at least one unmethylated CpG is administered in conjunction with another adjuvant, the CpG oligonucleotide can be administered before, after, and/or simultaneously with the other adjuvant. For instance, the combination of adjuvants may be. . . risk of infection from being infected. In cases where the combination of adjuvants is given without antigen, with repeated administrations, CpG oligonucleotides or one of the components in the combination may be given alone for one or more of the administrations.

DETD The CpG oligonucleotide containing at least one unmethylated CpG can have an additional efficacy (e.g., antisense) in addition to its ability to enhance antigen-specific immune responses.

DETD In addition to the use of the combination of CpG oligonucleotides and non-nucleic acid adjuvants to induce an antigen specific immune response in humans, the methods of the preferred embodiments. . .

DETD . . . birds are housed in closed quarters, leading to the rapid spread of disease. Thus, it is desirable to administer the CpG oligonucleotide and the non-nucleic acid adjuvant of the invention to birds to enhance an antigen-specific immune response when antigen is present. The CpG oligonucleotide and the non-nucleic acid adjuvant of the invention could also be administered to birds without antigen to protect against. . .

DETD . . . may be administered subcutaneously, by spray, orally, intraocularly, intratracheally, nasally, in ovo or by other methods described herein. Thus, the CpG oligonucleotide and non-nucleic acid adjuvant of the invention can be administered to birds and other non-human vertebrates using routine vaccination. . .

DETD . . . the invention can be used to protect against infection in livestock, such as cows, horses, pigs, sheep, and goats. The CpG oligonucleotide and the non-nucleic acid adjuvant of the invention could also be administered with antigen for antigen-specific protection of long. . .

DETD . . . method for immunizing an infant by administering to an infant an antigen and an oligonucleotide containing at least one unmethylated CpG dinucleotide in an effective amount for inducing cell mediated immunity in the infant. In some embodiments the infant is also. . .

DETD . . . in 10-15% of individuals infected as adolescents or adults, but 90-95% for those infected (either vertically or horizontally) as infants. CpG oligonucleotides may be used, according to the invention, to reduce this further owing to a more rapid appearance and higher. . .

DETD . . . expression of a particular cytokine when higher levels are desired. Modulation of a particular cytokine can occur locally or systemically. CpG oligonucleotides can directly activate macrophages and dendritic cells to secrete cytokines. No direct activation of proliferation or cytokine secretion by. . . Cytokine profiles determine T cell regulatory and effector functions in immune responses. In general, Th1-type cytokines are induced, thus the **immunostimulatory** nucleic acids promote a Th1 type antigen-specific immune response including cytotoxic T-cells.

DETD . . . for inducing a Th1 immune response. The combination of adjuvants includes at least one oligonucleotide containing at least one umnethylated CpG dinucleotide and at least one non-nucleic acid adjuvant. It was not previously known that when CpG was combined with

a non-nucleic acid adjuvant, as described above, that the combination would produce an immune response with a. . . by the combination of adjuvants is synergistic. Another aspect of the invention is to induce a Th response by using **CPG** with a non-nucleic acid adjuvant that by itself induces a Th2 response.

DETD . . . Adjuvants that induce Th1 responses include but are not limited to MPL, MDP, ISCOMS, IL-12, IFN-γ, and SB-AS2. When the **CpG** oligonucleotide is administered with a non-nucleic acid adjuvant the combination of adjuvants causes a commitment to a Th1 profile, that neither the adjuvant nor the **CpG** oligonucleotide is capable of producing on its own. Furthermore, if the non-nucleic acid adjuvant on its own induces a Th2 response, the addition of **CpG** oligonucleotide can overcome this Th2 bias and induce a Th1 response that may be even more Th1-like than with **CpG** alone.

DETD . . . Let. 29:2619-2622, 1988). These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, **CpG** dinucleotides can be produced on a large scale in plasmids, (see Sambrook, T., et al., Molecular Cloning: A Laboratory Manual,. . .

DETD Nucleic acids containing an appropriate unmethylated **CpG** can be effective in any mammal, preferably a human. Different nucleic acids containing an unmethylated **CpG** can cause optimal immune stimulation depending on the mammalian species. Thus an oligonucleotide causing optimal stimulation in humans may not. . .

DETD The **CpG** ODN of the invention stimulate cytokine production (e.g., IL-6, IL-1 2, IFN-γ, TNF-α and GM-CSF) and B-cell proliferation in PBMC's. . .

DETD . . . GGGGTCAGTCGTGACGGGG; (SEQ ID NO: 47)
GCTAGACGTTAGTGT; (SEQ ID NO: 48)
TCCATGTCGTTCCTGATGCT; (SEQ ID NO: 49)
ACCATGGACGATCTGTTTCCCCTC; (SEQ ID NO: 50)
TCTCCCAGCGTGCGCCAT; (SEQ ID NO: 51)
ACCATGGACGAACTGTTTCCCCTC; (SEQ ID NO: 52)
ACCATGGACGAGCTGTTTCCCCTC; (SEQ ID NO: 53)
ACCATGGACGACCTGTTTCCCCTC; (SEQ ID NO: 54)
ACCATGGACGTACTGTTTCCCCTC;. . . TGTCGTTGTCGTTGTCGTT; (SEQ ID NO: 82)
TCGTCGTCGTCGTT; (SEQ ID NO: 83)
TGTCGTTGTCGTT; (SEQ ID NO: 84)
TCCATAGCGTTCCTAGCGTT; (SEQ ID NO: 85)
TCCATGACGTTCCTGACGTT; (SEQ ID NO: 86)
GTCGYT; (SEQ ID NO: 87)
TGTCGYT; (SEQ ID NO: 88)
AGCTATGACGTTCCAAGG; (SEQ ID NO: 89)
TCCATGACGTTCCTGACGTT; (SEQ ID NO: 90)
ATCGACTCTCGAACGTTCTC; (SEQ ID NO: 91)
TCCATGTCGGTCCTGACGCA; (SEQ ID NO: 92)
TCTTCGAT; (SEQ ID NO: 93)
ATAGGAGCTCCAACGTTCTC;. . .

DETD Preferred **CpG** ODN can effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml of. . . indication. These cytokines can be measured by assays well known in the art. The oligonucleotides listed above or other preferred **CpG** ODN can effect at least about 10%, more preferably at least about 15% and most preferably at least about 20%. . .

DETD The term "effective amount" of a **CpG** oligonucleotide refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of an oligonucleotide containing at least one unmethylated **CpG** and a non-nucleic acid adjuvant for treating an infectious disorder is that amount necessary to cause the development of an. . . amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular **CpG** oligonucleotide being administered (e.g. the number of unmethylated **CpG** motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or. . .

DETD The use of **CpG** ODN as an adjuvant alone or in combination with other adjuvants was evaluated. The hepatitis B virus surface antigen (HBsAg). . .

DETD . . . cells (Medix Biotech #ABH0905). This was diluted in saline for use without adjuvant. HBsAg was also formulated with alum and/or **CpG** ODN as adjuvant. HBsAg protein was mixed with aluminum hydroxide (Alhydrogel 85, [Al_{203}], Superfos Biosector, Vedbaek, Denmark) in the same. . .

DETD For groups treated with **CpG** ODN, an appropriate volume of synthetic oligodeoxynucleotide (ODN # 1826) of the sequence **TCCATGACGTTCCTGACGTT** (SEQ ID NO. 86) synthesized with a phosphorothioate backbone (Oligos Etc. & Oligo Therapeutics, Wilsonville, Oreg.) was added alone or. . . injection into the left tibialis anterior (TA) muscle of 1 or 2 ug HBsAg, without or with adjuvant (alum and/or **CpG** ODN), in 50 1 vehicle. When **CpG** DNA was added, each animal received a total of 1,

10, 100 or 500 µg ODN. Newborn mice were immunized. . .

DETD . . . mg Al3+/mg HbsAg). Each monkey received an injection of 0.5 ml containing 10 µg HbsAg. For some monkeys, 500 µg **CpG** ODN 1968 (TCGTCGCTGTTGTCGTTTCTT) (SEQ ID NO 72) was added to the vaccine formulation.

DETD . . . into the anterior thigh muscle of HBsAg *ay subtype, 20 µg/ml) combined with alum (25 mg Al3+/mg HBsAg), combined with **CpG**. **CpG** ODN 2006 (TCGTCGTGTCGTGTCGTT) (SEQ ID NO 77) was added to the vaccine formulation. Each orangutan received an injection of 1.0 ml containing 20 µg HBsAg with alum (500 µg), **CpG** oligonucleotide (1 mg) or both adjuvants.

DETD Comparison of **CpG** ODN and Non-nucleic Acid Adjuvants with HBsAg Subunit Vaccine

DETD . . . (i) alone, (ii) mixed with alum, (iii, iv, v, vi, vii) mixed with 0.1, 1, 10, 100 or 500 µg **CpG** ODN, or (viii, ix, x, xi, xii) mixed with both alum and 0.1, 1, 10, 100 or 500 µg **CpG** ODN. These mice were bled at 1, 2, 4 and 8 weeks after immunization and the plasma was assayed for. . .

DETD . . . of mice (n=5) were immunized with HBsAg (1 µg) alone, with alum (25 µg Al3+), with one of several different **CpG** and non-**CpG** control oligonucleotides of different backbones (10 µg), or with both alum and an oligonucleotide.

DETD Other groups of mice (n=5) were immunized as above (except only the 10 µg dose of **CpG** ODN was used) and boosted with the identical or a different formulation at 8 weeks, then spleens were removed 2. . .

DETD . . . mice were immunized with HBsAg (1 µg) and one of the following non-nucleic acid adjuvants alone or in combination with **CpG** ODN (10 µg): monophosphoryl lipid A (MPL, 50 µg, Ribi); Freund's Complete Adjuvant (CFA; 1:1 v/v); Freund's Incomplete Adjuvant (IFA;. .

.

DETD . . . 3, 7 or 14 days were injected with (i, ii, iii) a total of 1 µg HBsAg with alum, with **CpG** ODN 1826 (10 µg) or with both alum and **CpG** ODN, or with (iv) an HBsAg-expressing DNA vaccine (1-µg pCMV-S). Plasma was obtained at 4, 8, 12 and 16 weeks. . .

DETD Immunization of Cynomolgus Monkeys with HBsAg and Alum or Alum+**CpG** ODN

DETD . . . (HBsAg at 20 mg/ml adsorbed to alum, 25 mg Al3+/mg HBsAg) to which had been added saline (0.1 ml) or **CpG** ODN 2006 (500 µg in 0.1 ml, SEQ #77). Monkeys were bled at 2, 8, 10, 12 and 14 weeks. . .

DETD Immunization of Orangutans with HBsAg and Alum or **CpG** ODN or Alum+**CpG** ODN

DETD . . . and 4 weeks with 1 ml of vaccine containing HBsAg (10 µg) plus (i) alum (25 mg Al3+/mg HBsAg)(n=13), (ii) **CpG** ODN 2006 (SEQ# 77) (m=24) or (iii) alum plus **CpG** ODN (n=14). Animals were bled at 4.8 and 12 weeks and plasma was evaluated for anti-HBs titers (mIU/ml).

DETD Synergy of **CpG** ODN with Alum as Adjuvant for HBV Subunit Vaccine in Mice

DETD . . . <100) by 4 weeks. These titers were about 10-fold higher with the addition of alum as adjuvant, 60-fold higher with **CpG** ODN and more than 500-fold higher with both alum and **CpG** ODN. At later time points, the highest peak titers were with HBsAg/alum/**CpG**, the second highest with HBsAg/**CpG**, then HBsAg/alum (FIG. 1).

DETD . . . the immune system is even less mature than a newborn human, 10% and 0% of mice seroconverted with alum and **CpG** ODN alone respectively, but 75% serocoinverted when **CpG** ODN and alum were used together. In 7 day old mice, which have an immune system similar in maturity to that of a newborn human, seroconversion for alum, **CpG** or the combination was 11%, 22% and 1 00% respectively (FIG. 8). Furthermore, in these 7 day old mice, antibody. . .

DETD When used alone or combined with alum, there is a dose-response for **CpG** ODN with the best results being obtained with an intermediate dose (10 µg) and no further or only relatively small. . .

DETD When a large panel of ODN is compared for adjuvant activity it can be seen that **CPG** ODN with a nuclease-resistant phosphorothioate backbone have the best adjuvant effects (FIG. 3). There was very little or no adjuvant activity of non-**CpG** control ODN with a phosphorothioate backbone, or of **CpG** ODN with a chimeric or phosphodiester backbone. However, for those phosphorothioate **CpG** ODN that did not have adjuvant effect, all exhibited a synergistic effect with alum. In general, antibody titers with combined alum and **CpG** ODN were 10 to 100-fold higher than with **CpG** ODN and/or 100 to 1000-fold higher than with alum alone (FIG. 3).

DETD . . . with HBsAg and no adjuvant, and were completely lost with the addition of alum. CTL were augmented equally with both **CpG** ODN as with combined alum and **CpG** ODN (FIG. 1). A synergy for CTL responses could be seen with prime-boost strategies, in that priming with **CpG** ODN and boosting with alum gave better CTL than priming and boosting with **CpG** alone (FIG. 4) (Note: use of alum alone completely abrogates the CTL response).

DETD A synergistic action of **CpG** ODN and alum on CTL was very evident with immunization of young (7 day old) mice. In this case, neither alum nor

CpG ODN used alone induced significant levels of HBsAg-specific CTL, but when used together there wre very strong CTL were observed. . .

DETD Thus, **CpG** ODN is superior to alum for both humoral and cell-mediated responses, when each is used alone as adjuvant with the. . . action such that antibody and CTL activity are stronger than when either adjuvant is used alone. These results indicate that **CpG** ODN could be used to replace alum in vaccine formulations, which could be desirable to avoid associated side-effects due to. . . not possible to use alum because chemical interactions interfere with the efficacy of the vaccine. This should not occur with **CpG** ODN. Of even greater interest is the strong synergistic response when **CpG** ODN and alum are used together as adjuvants. This could allow better immune responses with lower or fewer doses of antigen. There is a fairly flat dose response to **CpG** ODN whether or not alum is present, indicating that a wide range of **CpG** ODN could be useful to adjuvant vaccines in humans.

DETD Synergy of **CpG** ODN with Other Non-nucleic Acid Adjuvants for HBV Subunit Vaccine in Mice.

DETD As discussed above, **CpG** ODN alone gave 8-fold higher antibody titers than alum, the only adjuvant currently licensed for human use. It also produces. . . in a dose of five times less than that of MPL. There was, as discussed above, a strong synergy with **CpG** ODN and alum, but in contrast no such synergy was seen with MPL and alum. Owing to the strong synergistic effect of alum and **CpG** ODN, this combination of adjuvants is even better than Freund's complete adjuvant (FCA) for inducing antibodies in mice (FIG. 5). . .

DETD The synergy seen with **CpG** ODN and alum, was also seen with **CpG** ODN combined with other adjuvants. When used alone, **CpG** ODN and Freund's incomplete adjuvant (FIA, a type of mineral oil) induced similar antibody titers, but when used together the anti-HBs titers were more than 50-fold higher than with either adjuvant alone. Indeed, the combination of **CpG** ODN and FIA was even better than FCA (FIG. 6).

DETD Similarly, **CpG** ODN and MPL alone gave equally high antibody titers, but when used together the titers were about 4-times higher than with either adjuvant alone (FIG. 7). While the synergistic response with **CpG** and MPL was not as marked with respect to overall antibody titers, it was very pronounced with respect to the. . .

DETD Dominance and Synergy of **CpG** ODN with Alum for Induction of a Th1-type immune response including CTL

DETD . . . to Th1 -type cytokines such as IL-12 and IFN-γ. Rather, almost all (>99%) antibodies were of the IgG1 isotype IgG2a:IgG1=0.01. **CpG** ODN induces significantly more IgG2a antibodies, such that they made up at least 50% of the total IgG (IgG (IgG2a:IgG1=1.4). The combination of alum and **CpG** ODN induce an equally strong Th1 response as **CpG** ODN alone (IgG2a:IgG1=1.0), despite the extremely strong Th2-bias of alum (FIG. 5). Similarly CTL responses with **CpG** ODN plus alum were as strong as those with **CpG** ODN alone, despite the fact that the Th2-bias of alum resulted in a complete loss of CTL when alum was.

DETD The strong Th1 bias with **CpG** is even more evident in neonatal and young mice, which are known to naturally have a strong Th2-bias to their immune system. In this case, neither alum nor **CpG** ODN on their own induced detectable IgG2a, indicating a very poor or absent Th1 response. Remarkably, when used together, **CpG** ODN and alum induced high levels of Ig G2a antibodies, which were now the predominant form of IgG (FIG. 10). Similarly, neither **CpG** ODN or alum induced significant levels of CTL in young mice, yet when used together there was a strong CTL. . .

DETD The strength of the Th1 influence of **CpG** ODN is seen not only by its ability to dominate over the Th2 effect of alum when they are co-administered,. . . owing to the strong Th2 bias of alum (FIGS. 1 and 4). However, in mice using alum at prime and **CpG** at boost, good CTL were induced, indicating the possibility of **CpG** to overcome a previously established Th2 response (FIG. 4).

DETD . . . of HBV-specific CTL is thought to contribute to the chronic carrier state. In contrast, one of the primary advantages of **CpG** DNA over alum as an adjuvant is the Th1-bias of the responses and thus the possibility to induce CTL. A striking finding from the present study is that **CpG** can completely counteract the Th2-bias of alum when the two adjuvants are delivered together, and in the case of immunization in early life, the combination can even give a more Th1 response than **CpG** ODN alone. This could allow one to capitalize on the strong synergistic action of the two adjuvants on the humoral. . .

DETD . . . the high hygiene level and rapid treatment of childhood infections (Cookson and Moffatt, 1997). Early exposure to bacterial DNA (and **immunostimulatory CpG** motifs) pushes the immune system away from Th2- and towards a Th1-type response and this may account for the lower. . . asthma in less developed countries, where there is a much higher frequency of upper respiratory infections during childhood. Addition of **CpG** ODN as adjuvant to all pediatric vaccines could re-establish a Th1-type response thereby reducing the incidence of

asthma.

DETD Synergy of **CpG** ODN with Other Adjuvants for Induction of a Th1-type Immune Responses

DETD The synergistic effect of **CpG** ODN on Th1 responses was also seen using other adjuvants. IFA on its own induces a very strong Th2-type response with virtually no IgG2a antibodies (IgG2a:IgG1=0.002) and **CpG** ODN on its own induces a moderate Th1 response (IgG2a:IgG1=1.4), but together the response was very strongly Th1 (IgG2a:IgG1=24.0). It. . .

DETD Similarly, **CpG** and MPL on their own are moderately Th1 (IgG2a:IgG1 ratios at 4 weeks are 1.4 and 1.9 respectively), but together. . .

DETD **CpG** ODN as Synergistic Adjuvant in Cynomolgus Monkeys

DETD **CpG** ODN, in combination with alum, also acts as a potent adjuvant to augment anti-HBs responses in Cynomolgus monkeys. Compared to responses obtained with the commercial HBV vaccine that contains alum, monkeys immunized with the commercial vaccine plus **CpG** ODN attained titers 50-times higher after prime and 10-times higher after boost (FIG. 14).

DETD **CpG** ODN as Synergistic Adjuvant to HBsAg in Hyporesponder Orangutans

DETD . . . 1988), only 0% and 15% of vaccinated orangutans have seroconverted by the same times. With the addition of 1 mg **CpG** ODN, this becomes 43% and 100% respectively. A synergistic response is seen even in these hyporesponders, because antibody levels and seroconversion rates are better with **CpG** ODN plus alum than with either adjuvant alone (FIG. 12).

. . . of a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated **CpG** dinucleotide and at least one non-nucleic acid adjuvant.

L17 ANSWER 5 OF 8 USPATFULL on STN

2001:79141 **Immunostimulatory** nucleic acid molecules.

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University of Iowa Research Foundation, Iowa City, IA, United States (U.S. corporation)Coley Pharmaceutical Group, Inc., Wellesley, MA, United States (U.S. corporation)The United States of America as represented by the Department of Health and Human Services, Washington, DC, United States (U.S. government)

US 6239116 B1 20010529

APPLICATION: US 1997-960774 19971030 (8)

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A method for inducing Il-6 in a subject comprising administering to the subject an effective amount to induce Il-6 in the subject of an **immunostimulatory** nucleic acid, having a sequence comprising: 5'X_1 X_2 CGX_3 X3' wherein C is unmethylated, wherein X_1, X_2 and X_3, X_4 are nucleotides, and wherein the 5' X_1 X_2 CGX_3 X_4 3' sequence is a non-palindromic sequence.

2. The method of claim 1, wherein the subject is human.

3. The method of claim 1, wherein the nucleic acid has 8 to 100 nucleotides.

4. The method of claim 1, wherein the nucleic acid backbone includes a phosphate backbone modification on the 5' inter-nucleotide linkages.

5. The method of claim 1, wherein the nucleic acid backbone includes a phosphate backbone modification on the 3' inter-nucleotide linkages.

6. The method of claim 1, wherein the nucleic acid includes a phosphate backbone modification.

7. The method of claim 1, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, GpT, GpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

8. The method of claim 1, wherein X_1 X_2 are GpA and X_3 X_4 are TpT.

9. The method of claim 1, wherein X_1 and X_2 are purines and X_3 and X_4 are pyrimidines.

10. The method of claim 1, wherein X_1 X_2 are GpA and X_3

and X_4 are pyrimidines.

11. The method of claim 1, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

12. The method of claim 1, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and N is a nucleic acid sequence composed of from about 2-25 nucleotides.

13. The method of claim 1, wherein the **immunostimulatory** nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCCATGTCGCTCCTGATGCT (SEQ ID NO:37); TCCATAACGTTCCTGATGCT (SEQ ID NO:2); TCCATGACGATCCTGATGCT (SEQ ID NO:87); TCCATGGCGGTCCTGATGCT (SEQ ID NO:34); TCCATGTCGGTCCTGATGCT (SEQ ID NO:28); TCCATAACGTCCCTGATGCT (SEQ ID NO:88); TCCATGTCGTTCCTGATGCT (SEQ ID NO:38); and TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:46).

14. A method of stimulating natural killer cell lytic activity comprising exposing a natural killer cell to an **immunostimulatory** nucleic acid to stimulate natural killer cell lytic activity, the **immunostimulatory** nucleic acid having a sequence comprising: 5'X_1 X_2 CGX_3 X3' wherein C is unmethylated, wherein X_1 X_2 and X_3 X_4 are nucleotides, and wherein the 5'X_1 X_2 CGX_3 X_4 3' sequence is a non-palindromic sequence.

15. The method of claim 14, wherein the nucleic acid has 8 to 100 nucleotides.

16. The method of claim 14, wherein the nucleic acid backbone includes a phosphate backbone modification on the 5' inter-nucleotide linkages.

17. The method of claim 14, wherein the nucleic acid backbone includes a phosphate backbone modification on the 3' inter-nucleotide linkages.

18. The method of claim 14, wherein the nucleic acid includes a phosphate backbone modification.

19. The method of claim 14, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

20. The method of claim 14, wherein X_1 X_2 are GpA and X_3 X_4 are TpT.

21. The method of claim 14, wherein X_1 and X_2 are purines and X_3 and X_4 are pyrimidines.

22. The method of claim 14, wherein X_1 X_2 are GpA and X_3 and X_4 are pyrimidines.

23. The method of claim 14, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

24. The method of claim 15, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and N is a nucleic acid sequence composed of from about 2-25 nucleotides.

25. The method of claim 14, wherein the **immunostimulatory** nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCGTCGTTGTCGTTGTCGTT (SEQ ID NO:47); TCCATGACGGTCCTGATGCT (SEQ ID NO:35); TCCATGACGATCCTGATGCT (SEQ ID NO:87); TCCATGACGCTCCTGATGCT (SEQ ID NO:89); TCCATGACGTTCCTGATGCT (SEQ ID NO:7); TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:46); TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO:49); GCGTGCGTTGTCGTTGTCGTT (SEQ ID NO:56); TGTCGTTTGTCGTTTGTCGTT(SEQ ID NO:48); TGTCGTTGTCGTTGTCGTT (SEQ ID NO:50); and TCGTCGTCGTCGTT (SEQ ID NO:51).

26. A method for inducing interferon-gamma in a subject to treat an immune system deficiency, comprising: administering to a subject having an immune system deficiency an effective amount to induce interferon-gamma production in the subject of an **immunostimulatory** nucleic acid, having a sequence comprising: 5'X_1 X_2 CGX_3 X_4 3' wherein C is unmethylated, wherein X_1 X_2 and X_3 X_4 are nucleotides, and wherein the sequence of the formula

X_1 X_2 CGX_3 X_4 is not palindromic.

27. The method of claim 26, wherein the nucleic acid has 8 to 100 nucleotides.

28. The method of claim 26, wherein the nucleic acid backbone includes a phosphate backbone modification on the 5' inter-nucleotide linkages.

29. The method of claim 26, wherein the nucleic acid backbone includes a phosphate backbone modification on the 3' inter-nucleotide linkages.

30. The method of claim 26, wherein the nucleic acid includes a phosphates backbone modification.

31. The method of claim 26, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

32. The method of claim 26, wherein X_1 X_2 are GpA and X_3 X_4 are TpT.

33. The method of claim 26, wherein X_1 and X_2 are purines and X_3 and X_4 are pyrimidines.

34. The method of claim 26, wherein X_1 X_2 are GpA and X_3 and X_4 are pyrimidines.

35. The method of claim 26, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

36. The method of claim 26, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and N is a nucleic acid sequence composed of from about 2-25 nucleotides.

37. A method for inducing Il-12 in a subject comprising: administering to the subject an effective amount to induce Il-12 in the subject, of an **immunostimulatory** nucleic acid having a sequence comprising: 5'X_1 X_2 CGX_3 X3' wherein C is unmethylated, wherein X_1, X_2, X_3, and X_4 are nucleotides, and wherein the sequence of the formula X_1 X_2 CGX_3 X_4 is not palindromic.

38. The method of claim 37, wherein the subject is human.

39. The method of claim 37, wherein the **immunostimulatory** nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCCATGTCGCTCCTGATGCT (SEQ ID NO:37); TCCATGACGATCCTGATGCT (SEQ ID NO:87); TCCATGGCGGTCCTGATGCT (SEQ ID NO:34); TCCATGTCGGTCCTGATGCT (SEQ ID NO:28); TCCATAACGTCCCTGATGCT (SEQ ID NO:88); TCCATGTCGTTCCTGATGCT (SEQ ID NO:38); and TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:46).

40. The method of claim 37, wherein the nucleic acid has 8 to 100 nucleotides.

41. The method of claim 37, wherein the nucleic acid backbone includes a phosphate backbone modification on the 5' inter-nucleotide linkages.

42. The method of claim 37, wherein the nucleic acid backbone includes a phosphate backbone modification on the 3' inter-nucleotide linkages.

43. The method of claim 37, wherein the nucleic acid includes a phosphate backbone modification.

44. The method of claim 37, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

45. The method of claim 37, wherein X_1 X_2 are GpA and X_3 X_4 are TpT.

46. The method of claim 37, wherein X_1 and X_2 are purines and X_3 and X_4 are pyrimidines.

47. The method of claim 37, wherein X_1 X_2 are GpA and X_3 and X_4 are pyrimidines.

48. The method of claim 37, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

49. The method of claim 37, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and N is a nucleic acid sequence composed of from about 2-25 nucleotides.

TI **Immunostimulatory** nucleic acid molecules

AI **US 1997-960774 19971030 (8)**

AB Nucleic acid sequences containing unmethylated **CpG** dinucleotides that modulate an immune response including stimulating a Th1 pattern of immune activation, cytokine production, NK lytic activity, and. . .

SUMM . . . present invention relates generally to oligonucleotides and more specifically to oligonucleotides which have a sequence including at least one unmethylated **CpG** dinucleotide which are **immunostimulatory**.

SUMM . . . cAMP response element, the CRE, the consensus form of which is the unmethylated sequence TGACGTC (binding is abolished if the **CpG** is methylated) (Iguchi-Ariga, S. M. M., and W. Schaffner: "**CpG** methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation". Genes & Develop. 3:612,. . .

SUMM The present invention is based on the finding that certain nucleic acids containing unmethylated cytosine-guanine (**CpG**) dinucleotides activate lymphocytes in a subject and redirect a subject's immune response from a Th2 to a Th1 (e.g. by. . . to produce Th1 cytokines, including IL-12, IFN-γ and GM-CSF). Based on this finding, the invention features, in one aspect, novel **immunostimulatory** nucleic acid compositions.

SUMM In one embodiment, the invention provides an isolated **immunostimulatory** nucleic acid sequence containing a **CpG** motif represented by the formula:

SUMM In another embodiment, the invention provides an isolated **immunostimulatory** nucleic acid sequence contains a **CpG** motif represented by the formula:

SUMM In another embodiment, autoimmune disorders are treated by inhibiting a subject's response to **CpG** mediated leukocyte activation. The invention provides administration of inhibitors of endosomal acidification such as bafilomycin a, chloroquine, and monensin to. . .

DRWD FIG. 1 B. Control phosphodiester oligodeoxynucleotide (ODN) 5' ATGGAAGGTCCAGTGTTCTC3' (SEQ ID No: 1) (.box-solid.) and two phosphodiester **CpG** ODN 5' ATCGACCTACGTGCGTTCTC3' (SEQ ID No: 2) (.diamond-solid.) and 5' TCCATAACGTTCCTGATGCT3' (SEQ ID No: 3) (.circle-solid.).

DRWD FIG. 1 C. Control phosphorothioate ODN 5' GCTAGATGTTAGCGT3' (SEQ ID No: 4) (.box-solid.) and two phosphorothioate **CpG** ODN 5' GAGAACGTCGACCTTCGAT3' (SEQ ID No: 5) (.diamond-solid.) and 5' GCATGACGTTGAGCT3' (SEQ ID No: 6) (.circle-solid.). Data present the mean±standard. . .

DRWD FIG. 2 is a graph plotting IL-6 production induced by **CpG** DNA in vivo as determined 1-8 hrs after injection. Data represent the mean from duplicate analyses of sera from two mice. BALB/c mice (two mice/group) were injected iv. with 100 μl of PBS (.quadrature.) or 200 μg of **CpG** phosphorothioate ODN 5' TCCATGACGTTCCTGATGCT3' (SEQ ID No: 7) (.box-solid.) or non-**CpG** phosphorothioate ODN 5' TCCATGAGCTTCCTGAGTCT3' (SEQ ID No: 8) (.diamond-solid.).

DRWD . . . periods after in vivo stimulation of BALB/c mice (two mice/group) injected iv with 100 μl of PBS, 200 μg of **CpG** phosphorothioate ODN 5' TCCATGACGTTCCTGATGCT3' (SEQ ID No: 7) or non-**CpG** phosphorothioate ODN 5' TCCATGAGCTTCCTGAGTCT3' (SEQ ID No: 8).

DRWD FIG. 4A is a graph plotting dose-dependent inhibition of **CpG**-induced IgM production by anti-IL-6. Splenic B-cells from DBA/2 mice were stimulated with **CpG** ODN 5' TCCAAGACGTTCCTGATGCT3' (SEQ ID No: 9) in the presence of the indicated concentrations of neutralizing anti-IL-6 (.diamond-solid.) or isotype control Ab (.circle-solid.) and IgM levels in culture supernatants determined by ELISA. In the absence of **CpG** ODN, the anti-IL-6 Ab had no effect on IgM secretion (.box-solid.).

DRWD FIG. 4B is a graph plotting the stimulation index of **CpG**-induced splenic B cells cultured with anti-IL-6 and **CpG** S-ODN 5' TCCATGACGTTCCTGATGCT3' (SEQ ID No: 7) (.diamond-solid.) or anti-IL-6 antibody only (.box-solid.). Data present the mean±standard deviation of triplicates.

DRWD . . . cells transfected with a promoter-less CAT construct (pCAT), positive control plasmid (RSV), or IL-6 promoter-CAT construct alone or cultured with **CpG** 5' CCATGACGTTCCTGATGCT3' (SEQ ID No: 7) or

non-**CpG** 5' TCCATGAGCTTCCTGAGTCT3' (SEQ ID No: 8) phosphorothioate ODN at the indicated concentrations. Data present the mean of triplicates.

DRWD FIG. 6 is a schematic overview of the immune effects of the **immunostimulatory** unmethylated **CpG** containing nucleic acids, which can directly activate both B cells and monocytic cells (including macrophages and dendritic cells) as shown. The **immunostimulatory** oligonucleotides do not directly activate purified NK cells, but render them competent to respond to IL-12 with a marked increase in their IFN-γ production. By inducing IL-12 production and the subsequent increased IFN-γ secretion by NK cells, the **immunostimulatory** nucleic acids promote a Th1 type immune response. No direct activation of proliferation of cytokine secretion by highly purified T cells has been found. However, the induction of Th1 cytokine secretion by the **immunostimulatory** oligonucleotides promotes the development of a cytotoxic lymphocyte response.

DRWD FIG. 7 is an autoradiograph showing NFkB mRNA induction in monocytes treated with E. coli (EC) DNA (containing unmethylated **CpG** motifs), control (CT) DNA (containing no unmethylated **CpG** motifs) and lipopolysaccharide (LPS) at various measured times, 15 and 30 minutes after contact.

DRWD . . . the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the **CpG** oligo (**TCCATGACGTTCCTGACGTT** SEQ ID No. 10) also showed an increase in the level of reactive oxygen species such that more than 50%. . .

DRWD . . . have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with **CpG** DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and. . .

DRWD . . . egg antigen "SEA" (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given **CpG** oligo (SEQ ID NO. 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation. . .

DRWD . . . and subsequently inhale SEA (open circle), many eosinophils are present in the lungs. However, when the mice are initially given **CpG** oligo (SEQ ID NO. 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation. . .

DRWD . . . inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a **CpG** oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes. . .

DRWD . . . or SEQ ID No. 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can actually redirect the cytokine response of the lung to production of IL-12, indicating a Th1 type of immune. . .

DRWD . . . or SEQ ID No. 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can also redirect the cytokine response of the lung to production of IFN-g, indicating a Th1 type of immune. . .

DETD An "**immunostimulatory** nucleic acid molecule" refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (i.e. "**CpG** DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates (e.g. has a mitogenic effect on, or induces or increases cytokine expression by) a vertebrate lymphocyte. An **immunostimulatory** nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have. . .

DETD In one preferred embodiment the invention provides an isolated immunostimulalory nucleic acid sequence containing a **CpG** motif represented by the formula:

DETD In another embodiment the invention provides an isolated **immunostimulatory** nucleic acid sequence contains a **CpG** motif represented by the formula:

DETD Preferably the **immunostimulatory** nucleic acid sequences of the invnetion include X_1 X_2 selected from the group consisting of GpT, GpG, GpA and ApA. . . selected from the group consisting of TpT, CpT and GpT (see for example, Table 5). For facilitating uptake into cells, **CpG** containing **immunostimulatory** nucleic acid molecules are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size (even many kb long) are **immunostimulatory** if sufficient **immunostimulatory** motifs are present, since such larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not. . . or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus mitogenic **CpG** motif is not a palindrome. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, where the oligonucleotide incorporates a phosphate backbone. . .

DETD Preferably the **immunostimulatory** **CpG** DNA is in the range of between

8 to 30 bases in size when it is an oligonucleotide. Alternatively, **CpG** dinucleotides can be produced on a large scale in plasmids, which after being administered to a subject are degraded into oligonucleotides. Preferred **immunostimulatory** nucleic acid molecules (e.g. for use in increasing the effectiveness of a vaccine or to treat an immune system deficiency. . .

DETD . . . useful as an adjuvant for use during antibody production in a mammal. Specific, but non-limiting examples of such sequences include: **TCCATGACGTTCCTGACGTT** (SEQ ID NO.10), GTCG(T/C)T and TGTCG(T/C)T. Furthermore, the claimed nucleic acid sequences can be administered to treat or prevent the symptoms of an asthmatic disorder by redirecting a subject's immune response from Th2 to Th1. An exemplary sequence includes **TCCATGACGTTCCTGACGTT** (SEQ ID NO.10).

DETD The stimulation index of a particular **immunostimulatory CpG** DNA can be tested in various immune cell assays. Preferably, the stimulation index of the **immunostimulatory CpG** DNA with regard to B-cell proliferation is at least about 5, preferably at least about 10, more preferably at least. . . treat an immune system deficiency by stimulating a cell-mediated (local) immune response in a subject, it is important that the **immunostimulatory CpG** DNA be capable of effectively inducing cytokine secretion by monocytic cells and/or Natural Killer (NK) cell lytic activity.

DETD Preferred **immunostimulatory CpG** nucleic acids should effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml. . . IL-6, 200 pg/ml IL-12, depending on the therapeutic indication, as determined by the assays described in Example 12. Other preferred **immunostimulatory CpG** DNAs should effect at least about 10%, more preferably at least about 15% and most preferably at least about 20%. . .

DETD . . . in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated **CpG** containing nucleic acid molecules that are tens to hundreds of kbs long are relatively resistant to in vivo degradation. For shorter **immunostimulatory** nucleic acid molecules, secondary structure can stabilize and increase their effect. For example, if the 3' end of a nucleic. . .

DETD . . . acid molecules (including phosphorodithioate-modified) can increase the extent of immune stimulation of the nucleic acid molecule, which contains an unmethylated **CpG** dinucleotide as shown herein. International Patent Application Publication Number: WO 95/26204 entitled "Immune Stimulation By Phosphorothioate Oligonucleotide Analogs" also reports on the non-sequence specific **immunostimulatory** effect of phosphorothioate modified oligonucleotides. As reported herein, unmethylated **CpG** containing nucleic acid molecules having a phosphorothioate backbone have been found to preferentially activate B-cell activity, while unmethylated **CpG** containing nucleic acid molecules having a phosphodiester backbone have been found to preferentially activate monocytic (macrophages, dendritic cells and monocytes) and NK cells. Phosphorothioate **CpG** oligonucleotides with preferred human motifs, are also strong activators of monocytic and NK cells.

DETD Certain Unmethylated **CpG** Containing Nucleic Acids Have B Cell Stimulatory Activity As Shown in vitro and in vivo

DETD . . . the other nonstimulatory control ODN. In comparing these sequences, it was discovered that all of the four stimulatory ODN contained **CpG** dinucleotides that were in a different sequence context from the nonstimulatory control.

DETD To determine whether the **CpG** motif present in the stimulatory ODN was responsible for the observed stimulation, over 300 ODN ranging in length from 5 to 42 bases that contained methylated, unmethylated, or no **CpG** dinucleotides in various sequence contexts were synthesized. These ODNs, including the two original "controls" (ODN 1 and 2) and two. . . then examined for in vitro effects on spleen cells (representative sequences are listed in Table 1). Several ODN that contained **CpG** dinucleotides induced B cell activation and IgM secretion; the magnitude of this stimulation typically could be increased by adding more **CpG** dinucleotides (Table 1; compare ODN 2 to 2a or 3D to 3Da and 3Db). Stimulation did not appear to result. . .

DETD Mitogenic ODN sequences uniformly became nonstimulatory if the **CpG** dinucleotide was mutated (Table 1; compare ODN 1 to 1a; 3D to 3Dc; 3M to 3Ma; and 4 to 4a) or if the cytosine of the **CpG** dinucleotide was replaced by 5-methylcytosine (Table 1; ODN 1b,2b,3Dd, and 3Mb). Partial methylation of **CpG** motifs caused a partial loss of stimulatory effect (compare 2a to 2c, Table 1). In contrast, methylation of other cytosines did not reduce ODN activity (ODN 1c, 2d, 3De and 3Mc). These data confirmed that a **CpG** motif is the essential element present in ODN that activate B cells.

DETD In the course of these studies, it became clear that the bases flanking the **CpG** dinucleotide played an important role in determining the

murine B cell activation induced by an ODN. The optimal stimulatory motif was determined to consist of a **CpG** flanked by two 5' purines (preferably a GpA dinucleotide) and two 3' pyrimidines (preferably a TpT or TpC dinucleotide). Mutations of ODN to bring the **CpG** motif closer to this ideal improved stimulation (e.g. Table 1, compare ODN 2 to 2e; 3M to 3Md) while mutations. . . Table 1, compare ODN 3D to 3Df; 4 to 4b, 4c and 4d). On the other hand, mutations outside the **CpG** motif did not reduce stimulation (e.g. Table 1, compare ODN 1 to id; 3D to 3Dg; 3M to 3Me). For. . .

DETD . . . 10 As. The effect of the G-rich ends is cis; addition of an ODN with poly G ends, but no **CpG** motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated **CpG** were found to be more **immunostimulatory**.

DETD . . . dev. derived from at least 3 separate experiments, and are compared to wells cultured with no added ODN.

ND = not done.

CpG dinucleotides are underlined.

Dots indicate identity; dashes indicate deletions.

Z indicates 5 methyl cytosine.

DETD TABLE 2

Identification of the optimal **CpG** motif for Murine IL-6 production and B cell activation

ODN	SEQUENCE (5'-3')	IL-6 (pg/ml)[a] CH12.LX	SPLENIC B CELL	SI[b]	IgM (ng/ml)[c]
512. . .		0.2	3534 ± 217		
1708	(SEQ ID No:106) .....CA..TG.....		ND	59 ± 3	1.5 ± 0.1
			466 ± 109		

Dots indicate identity; **CpG** dinucleotides are underlined; ND = not done

[a] The experiment was done at least three times with similar results. The level. . . CH12.LX and splenic B cells was ≤ 10 pg/ml. The IgM level of unstimulated culture was 547 ± 82 ng/ml. **CpG** dinucleotides are underlined and dots indicate identity.

[b] [^{3}H] Uridine uptake was indicated as a fold increase (SI: stimulation index) from unstimulated control (2322.67 ± 213.68 cpm). Cells were stimulated with 20 μM of various **CpG** O-ODN. Data present the mean ± SD of triplicates

[c] Measured by ELISA.

DETD . . . the subsequent fall in stimulation when purified B cells with or without anti-IgM (at a submitogenic dose) were cultured with **CpG** ODN, proliferation was found to synergistically increase about 10-fold by the two mitogens in combination after 48 hours. The magnitude. . .

DETD Cell cycle analysis was used to determine the proportion of B cells activated by **CpG**-ODN. **CpG**-ODN induced cycling in more than 95% of B cells. Splenic B lymphocytes sorted by flow cytometry into CD23- (marginal zone). . . as were both resting and activated populations of B cells isolated by fractionation over Percoll gradients. These studies demonstrated that **CpG**-ODN induce essentially all B cells to enter the cell cycle.

DETD **Immunostimulatory** Nucleic Acid Molecules Block Murine B Cell Apoptosis

DETD . . . are rescued from this growth arrest by certain stimuli such as LPS and by the CD40 ligand. ODN containing the **CpG** motif were also found to protect WEHI-231 from anti-IgM induced growth arrest, indicating that accessory cell populations are not required for the effect. Subsequent work indicates that **CpG** ODN induce Bcl-x and myc expression, which may account for the protection from apoptosis. Also, **CpG** nucleic acids have been found to block apoptosis in human cells. This inhibition of apoptosis is important, since it should enhance and prolong immune activation by **CpG** DNA.

DETD Identification of the optimal **CpG** motif for induction of Murine IL-6 and IgM secretion and B cell proliferation

DETD To evaluate whether the optimal B cell stimulatory **CpG** motif was identical with the optimal **CpG** motif for IL-6 secretion, a panel of ODN in which the bases flanking the **CpG** dinucleotide were progressively substituted was studied. This ODN panel was analyzed for effects on B cell proliferation, Ig production, and. . . using both splenic B cells and CH12.LX cells. As shown in Table 2, the optimal stimulatory motif contains an unmethylated **CpG** flanked by two 5' purines and two 3' pyrimidines. Generally a mutation of either 5' purine to pyrimidine or 3'. . . had less marked effects. Based on analyses of these and scores of other ODN, it was determined that the optimal **CpG** motif for induction of IL-6 secretion is TGACGTT, which is identical with the optimal mitogenic and IgM-inducing **CpG** motif (Table 2). This motif was more stimulatory than any of the palindrome containing sequences studied (1639, 1707 and 1708).

DETD Induction of Murine Cytokine Secretion by **CpG** motifs in Bacterial DNA or Oligonucleotides

DETD As described in Example 9, the amount of IL-6 secreted by spleen cells

after **CpG** DNA stimulation was measured by ELISA. T cell depleted spleen cell cultures rather than whole spleen cells were used for in vitro studies following preliminary studies showing, that T cells contribute little or nothing to the IL-6 produced by **CpG** DNA-stimulated spleen cells. As shown in Table 3, IL-6 production was markedly increased in cells cultured with E. coli DNA. . . response to bacterial DNA. To analyze whether the IL-6 secretion induced by E. coli DNA was mediated by the unmethylated **CpG** dinucleotides in bacterial DNA, methylated E. coli DNA and a panel of synthetic ODN were examined. As shown in Table 3, **CpG** ODN significantly induced IL-6 secretion (ODN 5a, 5b, 5c) while **CpG** methylated E. coli DNA, or ODN containing methylated **CpG** (ODN 5f) or no **CpG** (ODN 5d) did not. Changes at sites other than **CpG** dinucleotides (ODN 5b) or methylation of other cytosines (ODN 5g) did not reduce the effect of **CpG** ODN. Methylation of a single **CpG** in an ODN with three CpGs resulted in a partial reduction in the stimulation (compare ODN 5c to 5e; Table. . .

DETD TABLE 3

Induction of Murine IL-6 secretion by **CpG** motifs in bacterial DNA or oligonucleotides.

Treatment	IL-6 (pg/ml)
calf thymus DNA	≤10
calf thymus DNA + DNase	≤10
E. coli DNA	1169.5 ± 94.1
E. coli DNA + DNase	≤10
CpG methylated E. coli DNA	≤10
LPS	280.1 ± 17.1
Media (no DNA)	≤10

ODN

5a SEQ. ID. No:1 ATGGACTCTCCAGCGTTCTC 1096.4 ± 372.0

5b. . . or without enzyme treatment, or LPS (10 μg/ml) for 24 hr. Data represent the mean (pg/ml) ± SD of triplicates. **CpG** dinucleotides are underlined and dots indicate identity. Z indicates 5-methylcytosine.

DETD **CpG** motifs can be used as an artificial adjuvant

DETD . . . more acceptable side effects has led to the production of new synthetic adjuvants. The present invention provides the sequence 1826 **TCCATGACGTTCCTGACGTT** (SEQ ID NO: 10), which is an adjuvant including **CpG** containing nucleic acids. The sequence is a strong immune activating; sequence and is a superb adjuvant, with efficacy comparable or. . .

DETD Titration of induction of Murine IL-6 Secretion by **CpG** motifs

DETD Bacterial DNA and **CpG** ODN induced IL-6 production in T cell depleted murine spleen cells in a dose-dependent manner, but vertebrate DNA and non-**CpG** ODN did not (FIG. 1). IL-6 production plateaued at approximately 50 μg/ml of bacterial DNA or 40 μM of **CpG** O-ODN. The maximum levels of IL-6 induced by bacterial DNA and **CpG** ODN were 1-1.5 ng/ml and 2-4 ng/ml respectively. These levels were significantly greater than those seen after stimulation by LPS (0.35 ng/ml) (FIG. 1A). To evaluate whether **CpG** ODN with a nuclease-resistant DNA backbone would also induce IL-6 production, S-ODN were added to T cell depleted murine spleen cells. **CpG** S-ODN also induced IL-6 production in a dose-dependent manner to approximately the same level as **CpG** O-ODN while non-**CpG** S-ODN failed to induce IL-6 (FIG. 1C). **CpG** S-ODN at a concentration of 0.05 μM could induce maximal IL-6 production in these cells. This result indicated that the nuclease-resistant DNA backbone modification retains the sequence specific ability of **CpG** DNA to induce IL-6 secretion and that **CpG** S-ODN are more than 80-fold more potent than **CpG** O-ODN in this assay system.

DETD Induction of Murine IL-6 secretion by **CpG** DNA in vivo

DETD To evaluate the ability of bacterial DNA and **CpG** S-ODN to induce IL-6 secretion in vivo, BALB/c mice were injected iv. with 100 μg of E. coli DNA, calf thymus DNA, or **CpG** or non-stimulatory S-ODN and bled 2 hr after stimulation. The level of IL-6 in the sera from the E. coli. . . 13 ng/ml while IL-6 was not detected in the sera from calf thymus DNA or PBS injected groups (Table 4). **CpG** S-ODN also induced IL-6 secretion in vivo. The IL-6 level in the sera from **CpG** S-ODN injected groups was approximately 20 ng/ml. In contrast, IL-6 was not detected in the sera from non-stimulatory S-ODN stimulated. . .

DETD TABLE 4

Secretion of Murine IL-6 induced by **CpG** DNA stimulation in vivo.

Stimulant	IL-6 (pg/ml)
PBS	<50
E. coli DNA	13858 ± 3143
Calf Thymus DNA	<50
CpG S-ODN	20715 ± 606
non-**CpG** S-ODN	<50

Mice (2 mice/group) were i.v. injected with 100 μl of PBS, 200 μg of E. coli DNA or calf thymus DNA, or 500 μg of **CpG** S-ODN or non-**CpG** control S-ODN. Mice were bled 2 hr after injection and 1:10 dilution of

each serum was analyzed by IL-6 ELISA. Sensitivity limit of IL-6 ELISA was 5 pg/ml. Sequences of the **CpG** S-ODN is 5'GCATGACGTTGAGCT3' (SEQ. ID. No: 6) and of the non-stimulatory S-ODN is 5'GCTAGATGTTAGCGT3' (SEQ. ID. No: 49). Note that although

#there is a **CpG** in sequence 48, it is too close to the 3' end to effect stimulation, as explained herein. Data represent mean. . .

DETD Kinetics of Murine IL-6 secretion after stimulation by **CpG** motifs in vivo

DETD To evaluate the kinetics of induction of IL-6 secretion by **CpG** DNA in vivo, BALB/c mice were injected iv. with **CpG** or control non-**CpG** S-ODN. Serum IL-6 levels were significantly increased within 1 hr and peaked at 2 hr to a level of approximately 9 ng/ml in the **CpG** S-ODN injected group (FIG. 2). IL-6 protein in sera rapidly decreased after 4 hr and returned to basal level by 12 hr after stimulation. In contrast to **CpG** DNA stimulated groups, no significant increase of IL-6 was observed in the sera from the non-stimulatory S-ODN or PBS injected. . .

DETD Tissue distribution and kinetics of IL-6 mRNA expression induced by **CpG** moths in vivo

DETD As shown in FIG. 2, the level of serum IL-6 increased rapidly after **CpG** DNA stimulation. To investigate the possible tissue origin of this serum IL-6, and the kinetics of IL-6 gene expression in vivo after **CpG** DNA stimulation, BALB/c mice were injected iv with **CpG** or non-**CpG** S-ODN and RNA was extracted from liver, spleen, thymus, and bone marrow at various time points after stimulation. As shown. . . FIG. 3A, the level of IL-6 mRNA in liver, spleen, and thymus was increased within 30 min. after injection of **CpG** S-ODN. The liver IL-6 mRNA peaked at 2 hr post-injection and rapidly decreased and reached basal level 8 hr after. . . hr post-injection and then gradually decreased (FIG. 3A). IL-6 mRNA was significantly increased in bone marrow within 1 hr after **CpG** S-ODN injection but then returned to basal level. In response to **CpG** S-ODN, liver, spleen and thymus showed more substantial increases in IL-6 mRNA expression than the bone marrow.

DETD Patterns of Murine Cytokine Expression Induced by **CpG** DNA

DETD . . . within 30 minutes and the level of IL-6 increased strikingly within 2 hours in the serum of mice injected with **CpG** ODN. Increased expression of IL-12 and interferon gamma (IFN-γ) mRNA by spleen cells was also detected within the first two. . .

DETD TABLE 5

Induction of human PBMC cytokine secrtetion by **CpG** oligos

ODN	Sequence (5'-3')	IL-6[1]	TNF-α[1]	IFN-γ[1]	GM-CSF	IL-12
512 SEQ ID NO:28	TCCATGTCGGTCCTGATGCT	500	140	15.6	70	250
1637 SEQ ID NO:88	.....C.....	550	16	7.8.	. .	ID NO:3
1707 SEQ ID NO:88	.....A..TC.....	300	70	17	0	70
1708 SEQ ID NO:106	.....CA..TG.....	270	10	17	ND	10

dots indicate identity; **CpG** dinucleotides are underlined

[1] measured by ELISA using Quantikine kits from R&D Systems (pg/ml) Cells were cultured in 10% autologous serum. . .

DETD **CpG** DNA induces cytokine secretion by human PBMC, specifically monocytes

DETD . . . panels of ODN used for studying mouse cytokine expression were used to determine whether human cells also are induced by **CpG** motifs to express cytokine (or proliferate), and to identify the **CpG** motif(s) responsible. Oligonucleotide 1619 (GTCGTT; residues 6-11 of SEQ ID NO:105) was the best inducer of TNF-αand IFN-γ secretion, and. . . little induction of other cytokines. Thus, it appears that human lymphocytes, like murine lymphocytes, secrete cytokines differentially in response to **CpG** dinucleotides, depending on the surrounding bases. Moreover, the motifs that stimulate murine cells best differ from those that are most effective with human cells. Certain **CpG** oligodeoxynucleotides are poor at activating human cells (oligodeoxynucleotides 1707, 1708, which contain the palindrome forming sequences GACGTC; residues 6-11 of. . .

DETD . . . simply reflect a nonspecific death of all cell types Cytokine secretion in response to E. coli (EC) DNA requires unmethylated **CpG** motifs, since it is abolished by methylation of the EC DNA (next to the bottom row, Table 6). LPS contamination. . .

DETD TABLE 6

CpG DNA induces cytokine secretion by human PBMC

DNA	TNF-α(pg/ml)[1]	IL-6 (pg/ml)	IFN-γ (pg/ml)	RANTES (pg/ml)
EC DNA (50 μg/ml)	900	12,000.	. .	cytokine production under these

conditions was from monocytes (or other L-LME-sensitive cells).

[3] EC DNA was methylated using 2U/μg DNA of **CpG** methylase (New England Biolabs) according to the manufacturer's directions, and methylation

confirmed by digestion with Hpa-II and Msp-I. As a. . .

DETD . . . cytokine production in the PBMC treated with L-LME suggested that monocytes may be responsible for cytokine production in response to **CpG** DNA. To test this hypothesis more directly, the effects of **CpG** DNA on highly purified human, monocytes and macrophages was tested. As hypothesized, **CpG** DNA directly activated production of the cytokines IL-6, GM-CSF, and TNF-α by human macrophages, whereas non-**CpG** DNA did not (Table 7).

DETD TABLE 7

CpG DNA induces cytokine expression in purified human macrophages

	IL-6 (pg/ml)	GM-CSF (pg/ml)	TNF-α(pg/ml)
Cells alone	0	0	0

CT DNA (50 μg/ml). . .

DETD Biological Role of IL-6 in Inducing Murine IgM Production in Response to **CpG** Motifs

DETD The kinetic studies described above revealed that induction of IL-6 secretion, which occurs within 1 hr post **CpG** stimulation, precedes IgM secretion. Since the optimal **CpG** motif for ODN inducing secretion of IL-6 is the same as that for IgM (Table 2), whether the **CpG** motifs independently induce IgM and IL-6 production or whether the IgM production is dependent on prior IL-6 secretion was examined. The addition of neutralizing anti-IL-6 antibodies inhibited in vitro IgM production mediated by **CpG** ODN in a dose-dependent manner but a control antibody did not (FIG. 4A). In contrast, anti-IL-6 addition did not affect either the basal level or the **CpG**-induced B cell proliferation (FIG. 4B).

DETD Increased transcriptional activity of the IL-6promoter in response to **CpG** DNA

DETD The increased level of IL-6 mRNA and protein after **CpG** DNA stimulation could result from transcriptional or post-transcriptional regulation. To determine if the transcriptional activity of the IL-6 promoter was upregulated in B cells cultured with **CpG** ODN, a murine B cell line, WEHI-231, which produces IL-6 in response to **CpG** DNA, was transfected with an IL-6 promoter-CAT construct (pIL-6/CAT) (Pottratz, S. T. et al., 17B-estradiol) inhibits expression of human interleukin-6-promoter-reporter constructs by a receptor-dependent mechanism. J. Clin. Invest. 93:944). CAT assays were performed after stimulation with various concentrations of **CpG** or non-**CpG** ODN. As shown in FIG. 5, **CpG** ODN induced increased CAT activity in dose-dependent manner while non-**CpG** ODN failed to induce CAT activity. This confirms that **CpG** induces the transcriptional activity of the IL-6 promoter.

DETD Dependence of B cell activation by **CpG** ODN on the Number of 5' and 3' Phosphorothioate Internucleotide Linkages

DETD . . . DNA synthesis (by ^{3}H thymidine incorporation) in treated spleen cell cultures (Example 10). O-ODN (0/0 phosphorothioate modifications) bearing a **CpG** motif caused no spleen cell stimulation unless added to the cultures at concentrations of at least 10 μM (Example 10;.. . .

DETD Dependence of **CpG**-mediated lymphocyte activation on the type of backbone modification

DETD . . . result from the nuclease resistance of the former. To determine the role of ODN nuclease resistance in immune stimulation by **CpG** ODN, the stimulatory effects of chimeric ODN in which the 5' and 3' ends were rendered nuclease resistant with either. . .

DETD . . . while the S-ODN with the 3D sequence was less potent than the corresponding S-O-ODN (Example 10). In comparing the stimulatory **CpG** motifs of these two sequences, it was noted that the 3D sequence is a perfect match for the stimulatory motif in that the **CpG** is flanked by two 5' purines and two 3' pyrimidines. However, the bases immediately flanking the **CpG** in ODN 3D are not optimal; it has a 5' pyrimidine and a 3' purine. Based on further testing, it. . . for immune stimulation is more stringent for S-ODN than for S-O- or O-ODN. S-ODN with poor matches to the optimal **CpG** motif cause little or no lymphocyte activation (e.g. Sequence 3D). However, S-ODN with good matches to the motif, most critically at the positions immediately flanking the **CpG**, are more potent than the corresponding S-O-ODN (e.g. Sequence 3M, Sequences 4 and 6), even though at higher concentrations (greater. . .

DETD The increased B cell stimulation seen with **CpG** ODN bearing S or S_2 substitutions could result from any or All of the following effects: nuclease resistance, increased cellular. . . However, nuclease resistance can not be the only explanation, since the MP-O-ODN were actually less stimulatory than the O-ODN with **CpG** motifs. Prior studies have shown that ODN uptake by lymphocytes is markedly affected by the backbone chemistry (Zhao et al.,. . .

DETD Unmethylated **CpG** Containing Oligos Have NK Cell Stimulatory Activity

DETD Experiments were conducted to determine whether **CpG** containing oligonucleotides stimulated the activity of natural killer (NK) cells in addition to B cells. As shown in Table 8, a marked induction of NK activity among spleen cells cultured with **CpG** ODN 1 and 3Dd was

observed. In contrast, there was relatively no induction in effectors that had been treated with non-CpG control ODN.

DETD TABLE 8

Induction Of NK Activity By CpG Oligodeoxynucleotides (ODN)

	% YAC-1 Specific Lysis* Effector: Target			% 2C11 Specific Lysis Effector: Target	
ODN	50:1		100:1	50:1	100:1
None	-1.1	-1.4	15.3	16.6	
1	16.1		24.5	38.7	47.2
3Dd	17.1		27.0	37.0	40.0
non-CpG ODN	-1.6	-1.7	14.8	15.4	

DETD Induction of NK activity by DNA containing CpG motifs, but not by non-CpG DNA

DETD . . . 9). To determine whether the stimulatory activity of bacterial DNA may be a consequence of its increased level of unmethylated CpG dinucleotides, the activating properties of more than 50 synthetic ODN containing unmethylated, methylated, or no CpG dinucleotides was tested. The results, summarized in Table 9, demonstrate that synthetic ODN can stimulate significant NK activity, as long as they contain at least one unmethylated CpG dinucleotide. No difference was observed in the stimulatory effects of ODN in which the CpG was within a palindrome (such as ODN 1585, which contains the palindrome AACGTT) from those ODN without palindromes (such as 1613 or 1619), with the caveat that optimal stimulation was generally seen with ODN in which the CpG was flanked by two 5' purines or a 5' GpT dinucleotide and two 3' pyrimidines. Kinetic experiments demonstrated that NK. . . of the ODN. The data indicates that the murine NK; response is dependent on the prior activation of monocytes by CpG DNA, leading to the production of IL-12, TNF-α, and IFN-α/b (Example 11).

DETD TABLE 9

Induction of NK Activity by DNA Containing CpG Motifs but not by Non-CpG DNA

DNA or Cytokine Added			LU/10⁶ Mouse Cells	Human Cells
Expt. 1 None			0.00	0.00
IL-2			16.68	15.82
E.Coli. DNA			7.23	5.05
. . . No.42) 5.22				
1769	-------Z-----	(SEQ ID No.52)	0.02	ND
1619	TCCATGTCGTTCCTGATGCT	(SEQ ID No:38)	3.35	
1765	-------Z---------	(SEQ ID No.53)	0.11	

CpG dinucleotides in ODN sequences are indicated by underlying; Z indicates methylcytosine. Lower case letters indicate nuclease resistant phosphorothioate modified internucleotide. . .

DETD From all of these studies, a more complete understanding of the immune, effects of CpG DNA has been developed, which is summarized in FIG. 6.

DETD Immune activation by CpG motifs may depend on bases flanking the CpG, and the number and spacing of the CpGs present within an ODN. Although a single CpG in an ideal base context can be a very strong and useful immune activator, superior effects can be seen with ODN containing several CpGs with the appropriate spacing and flanking bases. For activation of murine B cells, the optimal CpG motif is TGACGTT; residues 10-17 of SEQ ID NO:70.

DETD . . . ODN sequences for stimulation of human cells by examining the effects of changing the number, spacing, and flanking bases of CpG dinucleotides.

DETD Identification of phosphorothioate ODN with optimal CpG motifs for activation of human NK cells

DETD . . . cellular uptake (Krieg et al., Antisense Res. Dev. 6:133, 1996.) and improved B cell stimulation if they also have a CpG motif. Since NK activation correlates strongly with in vivo adjuvant effects, the identification of phosphorothioate ODN that will activate human. .

DETD The effects of different phosphorothioate ODNs--containing CpG dinucleotides in various base contexts--on human NK activation (Table 10) were examined. ODN 1840, which contained 2 copies of the. . . 10). To further identify additional ODNs optimal for NK activation, approximately one hundred ODN containing different numbers and spacing of CpG motifs, were tested with ODN 1982 serving as a control. The results are shown in Table 11.

DETD . . . ODNs began with a TC or TG at the 5' end, however, this requirement was not mandatory. ODNs with internal CpG motifs (e.g. ODN 1840) are generally less potent stimulators than those in which a GTCGCT motif (residues 3-8 of SEQ. . . in which only one of the motifs had the addition of the spacing two Ts. The minimal acceptable spacing between CpG motifs is one nucleotide as long as the ODN has two pyrimidines (preferably T) at the 3' end (e.g., ODN. . . T also created a reasonably strong inducer of NK activity (e.g., ODN 2016). The

choice of thymine (T) separating consecutive **CpG** dinucleotides is not absolute, since ODN 2002 induced appreciable NK activation despite the fact that adenine (A) separated its CpGs (i.e., CGACGTT; residues 14-20 of SEQ ID NO:82). It should also be noted that ODNs containing no **CpG** (e.g., ODN 1982), runs of CpGs, or CpGs in bad sequence contexts (e.g., ODN 2010) had no stimulatory effect on. . .

DETD TABLE 10

ODN induction of NK Lytic Activity (LU)

ODN cells alone	Sequence (5'-3')	LU 0.01	
1754	ACCATGGACGATCTGTTTCCCCTC	0.02	SEQ ID NO: 59
1758	**TCTCCCAGCGTGCGCCAT**	0.05	SEQ ID NO: 45
1761	TACCGCGTGCGACCCTCT	0.05	SEQ ID NO: 60
1776	ACCATGGACGAACTGTTTCCCCTC	0.03	SEQ ID NO: 61
1777	ACCATGGACGAGCTGTTTCCCCTC	0.05	SEQ. . .

DETD TABLE 11

Induction of NK LU by Phoshorothioate **CpG** ODN with Good Motifs

ODN[1] cells alone	sequence (5'-3')	SEQ ID NO:	expt. 1 0.00	expt. 2 1.26	expt. 3 0.46
1840	TCCATGTCGTTCCTGTCGTT	73. . .			

(SEQ ID NO:83); Z = 5-methyl cytosine at residues 8 and 17; LU is lytic units; ND = not done; **CpG** dinucleotides are underlined for clarity

DETD Identification of phosphorothioate ODN with optimal **CpG** motifs for activation of human B cell proliferation

DETD The ability of a **CpG** ODN to induce B cell proliferation is a good measure of its adjuvant potential. Indeed, ODN with strong adjuvant effects generally also induce B cell proliferation. To determine whether the optimal **CpG** ODN for inducing B cell proliferation are the same as those for inducing NK cell activity, similar panels of ODN. . .

DETD TABLE 12

Induction of human B cell proliferation by Phosphorothioate **CpG** ODN

ODN	sequence (5' 3')	SEQ ID NO:	Stimulation Index[1L] expt. 1	expt. 2	expt. 3	expt. 4	expt. 5	expt.. . .

DETD The ability of a **CpG** ODN to induce IL-12 secretion is a good measure of its adjuvant potential, especially in terms of its ability to. . . IL-12 secretion from human PBMC in vitro (Table 13) was examined. These experiments showed that in some human PBMC, most **CpG** ODN could induce IL-12 secretion (e.g., expt. 1). However, other donors responded to just a few **CpG** ODN (e.g., expt. 2). ODN 2006 was a consistent inducer of IL2 secretion from most subjects (Table 13).

DETD TABLE 13

Induction of human IL-12 secretion by Phosphorothioate **CpG** ODN

ODN[1] cells alone	sequence (5'-3')	SEQ ID NO	IL-12 (pg/ml) expt. 1	expt. 2
			0	0
1962	TCCTGTCGTTCCTTGTCGTT	52	19	0
1965	TCCTGTCGTTTTTTGTCGTT	53. . .		

DETD As shown in FIG. 6, **CpG** DNA can directly activate highly purified B cells and monocytic cells. There are many similarities in the mechanism through which **CpG** DNA activates these cell types. For example, both require NFkB activation as explained further below.

DETD In further studies of different immune effects of **CpG** DNA, it was found that there is more than one type of **CpG** motif. Specifically, oligo 1668, with the best mouse B cell motif, is a strong inducer of both B cell and. . .

DETD TABLE 14

Different **CpG** motifs stimulate optimal murine B cell and NK activation

ODN	Sequence		B cell activation[1]	NK activation[2]
1668	TCCATGACGTTCCTGATGCT	(SEQ.ID.NO:7)	42,849	2.52
1758	**TCTCCCAGCGTGCGCCAT**	(SEQ.ID.NO.45)	1,747	6.66
NONE			367	0.00

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance. [1] Measured by ^{3}H. . .

DETD Teleological Basis of **Immunostimulatory**, Nucleic Acids

DETD Vertebrate DNA is highly methylated and **CpG** dinucleotides are underrepresented. However, the stimulatory **CpG** motif is common in microbial genomic DNA, but quite rare in vertebrate DNA. In addition, bacterial DNA has been reported. . . P. et al., J. Immunol. 147:1759 (1991)). Experiments further described in Example 3, in which methylation of bacterial DNA with **CpG** methylase was found to abolish mitogenicity, demonstrates that the difference in **CpG** status is the

cause of B cell stimulation by bacterial DNA. This data supports the following conclusion: that unmethylated **CpG** dinucleotides present within bacterial DNA are responsible for the stimulatory effects of bacterial DNA.

DETD Teleologically, it appears likely that lymphocyte activation by the **CpG** motif represents an immune defense mechanism that can thereby distinguish bacterial from host DNA. Host DNA, which would commonly be. . . regions and areas of inflammation due to apoptosis (cell death), would generally induce little or no lymphocyte activation due to **CpG** suppression and methylation. However, the presence of bacterial DNA containing unmethylated **CpG** motifs can cause lymphocyte activation precisely in infected anatomic regions, where it is beneficial. This novel activation pathway provides a rapid alternative to T cell dependent antigen specific B cell activation. Since the **CpG** pathway synergizes with B cell activation through the antigen receptor, B cells bearing antigen receptor specific for bacterial antigens would. . .

DETD . . . 35:647 (1992)), is likely triggered at least in part by activation of DNA-specific B cells through stimulatory signals provided by **CpG** motifs, as well as by binding of bacterial DNA to antigen receptors.

DETD . . . products released from dying bacteria that reach concentrations sufficient to directly activate many lymphocytes. Further evidence of the role of **CpG** DNA in the sepsis syndrome is described in Cowdery, J., et. al., (1996) The Journal of Immunology 156:4570-4575.

DETD Unlike antigens that trigger B cells through their surface Ig receptor, **CpG**-ODN did not induce any detectable Ca^{2+} flux, changes in protein tyrosine phosphorylation, or IP 3 generation. Flow cytometry with FITC-conjugated ODN with or without a **CpG** motif was performed sis described in Zhao, Q et al., (Antisense Research and Development 3:53-66 (1993)), and showed equivalent membrane binding, cellular uptake, efflux, and intracellular localization. This suggests that there may not be cell membrane proteins specific for **CpG** ODN. Rather than acting through the cell membrane, that data suggests that unmethylated **CpG** containing oligonucleotides require cell uptake for activity: ODN covalently linked to a solid Teflon support were nonstimulatory, as were biotinylated ODN immobilized on either avidin beads or avidin coated petri dishes. **CpG** ODN conjugated to either FITC or biotin retained full mitogenic properties, indicating no steric hindrance.

DETD Recent data indicate the involvement of the transcription factor NFkB as a direct or indirect mediator of the **CpG** effect. For example, within 15 minutes of treating B cells or monocytes with **CpG** DNA, the level of NFkB binding activity is increased (FIG. 7). However, it is not increased by DNA that does not contain **CpG** motifs. In addition, it was found that two different inhibitors of NFkB activation, PDTC and gliotoxin, completely block the lymphocyte stimulation by **CpG** DNA as measured by B cell proliferation or monocytic cell cytokine secretion, suggesting that NFkB activation is required for both. . .

DETD . . . various protein kinases, or through the generation of reactive oxygen species. No evidence for protein kinase activation induced immediately after **CpG** DNA treatment of B cells or monocytic cells have been found, and inhibitors of protein kinase A, protein kinase C, and protein tyrosine kinases had no effects on the **CpG** induced activation. However, **CpG** DNA causes a rapid induction of the production of reactive oxygen species in both B cells and monocytic cells, as. . . reactive oxygen species completely block the induction of NFkB and the later induction of cell proliferation and cytokine secretion by **CpG** DNA.

DETD Working backwards, the next question was how **CpG** DNA leads to the generation of reactive oxygen species so quickly. Previous studies by the inventors demonstrated that oligonucleotides and. . . rapidly become acidified inside the cell. To determine whether this acidification step may be important in the mechanism through which **CpG** DNA activates reactive oxygen species, the acidification step was blocked with specific inhibitors of endosome acidification including chloroquine, monensin, and. . . the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the **CpG** oligo also showed an increase in the level of reactive oxygen species such that more than 50% of the cells became positive (Panel D). However, cells treated with an oligonucleotide with the identical sequence except that the **CpG** was switched did not show this significant increase in the level of reactive oxygen species (Panel E).

DETD . . . have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with **CpG** DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and. . . This demonstrates that unlike the PMA plus ionomycin, the generation of reactive oxygen species following treatment of B cells with **CpG** DNA requires that the DNA undergo an acidification step in the endosomes. This is a completely novel mechanism of leukocyte activation. Chloroquine, monensin, and bafilomycin also appear to block the

activation of NFkB by **CpG** DNA as well as the subsequent proliferation and induction of cytokine secretion.

DETD Chronic Immune Activation by **CpG** DNA and Autoimmune Disorders

DETD B cell activation by **CpG** DNA synergizes with signals through the B cell receptor. This raises the possibility that DNA-specific B cells may be activated by the concurrent binding of bacterial DNA to their antigen receptor, and by the co-stimulatory **CpG**-mediated signals. In addition, **CpG** DNA induces B cells to become resistant to apoptosis, a mechanism thought to be important for preventing immune responses to self antigens, such as DNA. Indeed, exposure to bDNA can trigger anti-DNA Ab production. Given this potential ability of **CpG** DNA to promote autoimmunity, it is therefore noteworthy that patients with the autoimmune disease systemic lupus erythematosus have persistently elevated. . . . circulating plasma DNA which is enriched in hypomethylated CpGs. These findings suggest a possible role for chronic immune activation by **CpG** DNA in lupus etiopathogenesis.

DETD . . . While the therapeutic mechanism of these drugs has been unclear, they are known to inhibit endosomal acidification. Leukocyte activation by **CpG** DNA is not mediated through binding to a cell surface receptor, but requires cell uptake, which occurs via adsorptive endocytosis into an acidified chloroquine-sensitive intracellular compartment. This suggested the hypothesis that leukocyte activation by **CpG** DNA may occur in association with acidified endosomes, and might even be pH dependent. To test this hypothesis specific inhibitors of DNA acidicification were applied to determine whether B cells or monocytes could respond to **CpG** DNA if endosomal acidification was prevented.

DETD The earliest leukocyte activation event that was detected in response to **CpG** DNA is the production of reactive oxygen species (ROS), which is induced within five minutes in primary spleen cells and. . . cell lines. Inhibitors of endosomal acidification including chloroquine, bafilomycin A, and monensin, which have different mechanisms of action, blocked the **CpG**-induced generation of ROS, but had no effect on ROS generation mediated by PMA, or ligation of CD40 or IgM. These. . . diverse pathways. This ROS generation is generally independent of endosomal acidification, which is required only for the ROS response to **CpG** DNA. ROS generation in response to **CpG** is not inhibited by the NFκB inhibitor gliotoxin, confirming that it is not secondary to NFκB activation.

DETD To determine whether endosomal acidification of **CpG** DNA was also required for its other immune stimulatory effects were performed. Both LPS and **CpG** DNA induce similar rapid NFκB activation, increases in proto-oncogene mRNA levels, and cytokine secretion. Activation of NFκB by DNA depended on **CpG** motifs since it was not induced by bDNA treated with **CpG** methylase, nor by ODN in which bases were switched to disrupt the CpGs. Supershift experiments using specific antibodies indicated that the activated NFκB complexes included the p50 and p65 components. Not unexpectedly, NFκB activation in LPS- or **CpG**-treated cells was accompanied by the degradation of IκBα and IκBβ. However, inhibitors of endosomal acidification selectively blocked all of the **CpG**-induced but none of the LPS-induced cellular activation events. The very low concentration of chloroquine (<10 μM) that has been determined to inhibit **CpG**-mediated leukocyte activation is noteworthy since it is well below that required for antimalarial activity and oiler reported immune effects (e.g., 100-1000 μM). These experiments support the role of a pH-dependent signaling mechanism in mediating the stimulatory effects of **CpG** DNA.

DETD TABLE 15

Specific blockade of **CpG**-induced TNF-α and IL-12 expression by inhibitors of endosomal acidification or NFκB activation

	Inhibitors:								NAC
	TPCK		Gliotoxin		Bisgliotoxin				(50
	Bafilomycin		Chloroquine		Monensin				. . .
	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	TNF-α	TNF-α	
Medium	37	147	46	102	27	20	22	73	10
	24	17	41						
CpG	455	17,114	71	116	28	6	49	777	54
	23	31	441						
ODN									
LPS	901	22,485	1370	4051	1025	12418	491	4796.	. .

were cultured with or without the indicated inhibitors at the concentrations shown for 2 hr and then stimulated with the **CpG** oligodeoxynucleotide (ODN) 1826 (**TCCATGACGTTCCTGACGTT** SEQ ID NO:10) at 2 μM or LPS (10 μg/ml) for 4 hr (TNF-α or 24 hr (IL-12) at which. . . Immunol., 157, 5394-5402 (1996); Krieg, A. M, J Lab. Clin. Med., 128, 128-133 (1996). Cells cultured with ODN that lacked **CpG** motifs did not induce cytokine secretion. Similar specific

#inhibition of **CpG** responses was seen with IL-6 assays, and in experiments using primary spleen cells or the B cell lines CH12.LX and. . .

DETD Excessive immune activation by **CpG** motifs may contribute to the pathogenesis of the autoimmune disease systemic lupus erythematosus, which is associated with elevated levels of circulating hypomethylated **CpG** DNA. Chloroquine and related antimalarial compounds are effective therapeutic agents for the treatment of systemic lupus erythematosus and some other. . . mechanism of action has been obscure. Our demonstration of the ability of extremely low concentrations of chloroquine to specifically inhibit **CpG**-mediated leukocyte activation suggests a possible new mechanism for its beneficial effect. It is noteworthy that lupus recurrences frequently are thought. . . bDNA present in infected tissues can be sufficient to induce a local inflammatory response. Together with the likely role of **CpG** DNA as a mediator of the sepsis syndrome and other diseases our studies suggest possible new therapeutic applications for antimalarial. . .

DETD **CpG**-induced ROS generation could be an incidental consequence of cell activation, or a signal that mediates this activation. The ROS scavenger N-acetyl-L-cysteine (NAC) blocks **CpG**-induced NFκB activation, cytokine production, and B cell proliferation, suggesting a causal role for ROS generation in these pathways. These data. . . gliotoxin (0.2 μg/ml). Cell aliquots were then cultured as above for 10 minutes in RPMI medium with or without a **CpG** ODN (1826) or non-**CpG** ODN (1911) at 1 μM or phorbol myristate acetate (PMA) plus ionomycin (iono). Cells were then stained with dihydrorhodamine-123 and. . . 5394-5402 (1996); Krieg, A. M, J. Lab. Clin. Med., 128, 128-133 (1996)). J774 cells, a monocytic line, showed similar pH-dependent **CpG** induced ROS responses. In contrast, **CpG** DNA did not induce the generation of extracellular ROS, nor any detectable neutrophil ROS. These concentrations of chloroquine (and those used with the other inhibitors of endosomal acidification) prevented acidification of the internalized **CpG** DNA using fluorescein conjugated ODN as described by Tonkinson, et al., (Nucl. Acids Res. 22, 4268 (1994); A. M. Krieg,. . .

DETD While NFκB is known to be an important regulator of gene expression, it's role in the transcriptional response to **CpG** DNA was uncertain. To determine whether this NFκB activation was required for the **CpG** mediated induction of gene expression cells were activated with **CpG** DNA in the presence or absence of pyrrolidine dithiocarbamate (PDTC), an inhibitor of IκB phosphorylation. These inhibitors of NFκB activation completely blocked the **CpG**-induced expression of protooncogene and cytokine mRNA and protein, demonstrating the essential role of NFκB as a mediator of these events.. . . was cultured in the presence of calf thymus (CT), E. coli (EC), or methylated E. coli (mEC) DNA (methylated with **CpG** methylase as described[4]) at 5 μg/ml or a **CpG** oligodeoxynucleotide (ODN 1826; Table 15) or a non-**CpG** ODN (ODN 1745; TCCATGAGCTTCCTGAGTCT; SEQ ID NO:8) at 0.75 μM for 1 hr, following which the cells were lysed and. . . was determined by supershifting with specific Ab to p65 and p50 (Santa Cruz Biotechnology, Santa Cruz, Calif.). Chloroquine inhibition of **CpG**-induced but not LPS-induced NFκB activation was established using J774 cells. The cells were precultured for 2 hr in the presence or absence of chloroquine (20 μg/ml) and then stimulated as above for 1 hr with either EC DNA, **CpG** ODN, non-**CpG** ODN or LPS (1 μg/ml). Similar chloroquine sensitive **CpG**-induced activation of NFκB was seen in a B cell line, WEHI-231 and primary spleen cells. These experiments were performed three. . .

DETD It was also established that **CpG**-stimulated mRNA expression requires endosomal acidification and NFκB activation in B cells and monocytes. J774 cells (2×10^6 cells/ml) were cultured for. . . stimulated with the addition of E. coli DNA (EC; 50 μg/ml), calf thymus DNA (CT; 50 μg/ml), LPS (10 μg/ml), **CpG** ODN (1826; 1 μM), or control non-**CpG** ODN (1911; 1 μM) for 3 hr. WEHI-231 B cells (5×10^5 cells/ml) were cultured in the presence or absence of gliotoxin (0.1 μg/ml) or bisgliotoxin (0.1 μg/ml) for 2 hrs and then stimulated with a **CpG** ODN (1826), or control non-**CpG** ODN (1911; TCCAGGACTTTCCTCAGGTT; SEQ ID NO:107) at 0.5 μM for 8 hr. In both cases, cells were harvested and RNA. . .

DETD The results indicate that leukocytes respond to **CpG** DNA through a novel pathway involving the pH-dependent generation of intracellular ROS. The pH dependent step may be the transport or processing of the **CpG** DNA, the ROS generation, or some other event. ROS are widely thought to be second messengers in signaling pathways in. . .

DETD Presumably, there is a protein in or near the endosomes that specifically recognizes DNA containing **CpG** motifs and leads to the generation of reactive oxygen species. To detect any protein in the cell cytoplasm that may specifically bind **CpG** DNA, electrophoretic mobility shift assays (EMSA) were used with 5' radioactively labeled oligonucleotides with or without **CpG** motifs. A band was found that appears to represent a protein binding specifically to single stranded

oligonucleotides that have **CpG** motifs, but not to oligonucleotides that lack **CpG** motifs or to oligonucleotides in which the **CpG** motif has been methylated. This binding activity is blocked if excess of oligonucleotides that contain the NFkB binding site was. . . suggests that an NFkB or related protein is a component of a protein or protein complex that binds the stimulatory **CpG** oligonucleotides.

DETD . . . points where NFkB was strongly activated. These data therefore do not provide proof that NFkB proteins actually bind to the **CpG** nucleic acids, but rather that the proteins are required in some way for the **CpG** activity. It is possible that a CREB/ATF or related protein may interact in some way with NFkB proteins or other proteins thus explaining the remarkable similarity in the binding motifs for CREB proteins and the optimal **CpG** motif. It remains possible that the oligos bind to a CREB/ATF or related protein, and that this leads to NFkB. . .

DETD Alternatively, it is very possible that the **CpG** nucleic acids may bind to one of the TRAF proteins that bind to the cytoplasmic region of CD40 and mediate. . .

DETD Method for Making **Immunostimulatory** Nucleic Acids

DETD . . . described (Uhlmann, E. and Peyman, A. (1990) Chem. Rev. 90:544; Goodchild, J. (1990) Bioconjugate Chem. 1:165). 2'-O-methyl nucleic acids with **CpG** motifs also cause immune activation, as do ethoxy-modified **CpG** nucleic acids. In fact, no backbone modifications have been found that completely abolish the **CpG** effect, although it is greatly reduced by replacing the C with a 5-methyl C.

DETD Therapeutic Uses of **Immunostimulatory** Nucleic Acid Molecules

DETD Based on their **immunostimulatory** properties, nucleic acid molecules containing at least one unmethylated **CpG** dinucleotide can be administered to a subject in vivo to treat an "immune system deficiency". Alternatively, nucleic acid molecules containing at least one unmethylated **CpG** dinucleotide can be contacted with lymphocytes (e.g. B cells, monocytic cells or NK cells) obtained from a subject having an. . .

DETD As reported herein, in response to unmethylated **CpG** containing nucleic acid molecules, an increased number of spleen cells secrete IL-6, IL-12, IFN-γ, IFN-α, IFN-β, IL-1, IL-3, IL-10, TNF-α,. . .

DETD **Immunostimulatory** nucleic acid molecules can also be administered to a subject in conjunction with a vaccine to boost a subject's immune system and thereby effect a better response from the vaccine. Preferably the **immunostimulatory** nucleic acid molecule is administered slightly before or at the same time as the vaccine. A conventional adjuvant may optionally. . .

DETD . . . antigen encoded by the vaccine determines the specificity of the immune response. Second, if the backbone of the plasmid contains **CpG** motifs, it functions as an adjuvant for the vaccine. Thus, **CpG** DNA acts as an effective "danger signal" and causes the immune system to respond vigorously to new antigens in the area. This mode of action presumably results primarily from the stimulatory local effects of **CpG** DNA on dendritic cells and other "professional" antigen presenting cells, as well as from the co-stimulatory effects on B cells.

DETD **Immunostimulatory** oligonucleotides and unmethylated **CpG** containing vaccines, which directly activate lymphocytes and co-stimulate an antigen-specific response, are fundamentally different from conventional adjuvants (e.g. aluminum precipitates),. . .

DETD In addition, an **immunostimulatory** oligonucleotide can be administered prior to, along with or after administration of a chemotherapy or immunotherapy to increase the responsiveness. . . chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF. **CpG** nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Induction of NK activity and. . .

DETD Another use of the described **immunostimulatory** nucleic acid molecules is in desensitization therapy for allergies, which are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated **CpG** nucleic acids, are predominantly of a class called "Th1" which is most marked by a cellular immune response and is. . . are mediated by Th2 type immune responses and autoimmune diseases by Th1 immune response. Based on the ability of the **immunostimulatory** nucleic acid molecules to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of an **immunostimulatory** nucleic acid (or a vector containing a nucleic acid) alone or in conjunction with an allergen can be administered to. . .

DETD Nucleic acids containing unmethylated **CpG** motifs may also have significant therapeutic utility in the treatment of asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated. . .

DETD As described in detail in the following Example 12, oligonucleotides

containing an unethylated **CpG** motif (i.e., **TCCATGACGTTCCTGACGTT**; SEQ D NO. 10), but not a control oligonucleotide (TCCATGAGCTTCCTGAGTCT; SEQ ID NO 8) prevented the development of an inflammatory. . .

DETD For use in therapy, an effective amount of an appropriate **immunostimulatory** nucleic acid molecule alone or formulated as a delivery complex can be administered to a subject by any mode allowing. . .

DETD . . . to realize a desired biologic effect. For example, an effective amount of a nucleic acid containing at least one unmethylated **CpG** for treating an immune system deficiency could be that amount necessary to eliminate a tumor, cancer, or bacterial, viral or. . . such factors as the disease or condition being treated, the particular nucleic acid being administered (e.g. the number of unmethylated **CpG** motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or. . .

DETD . . . Table 1). In some experiments, culture supernatants were assayed for IgM by ELISA, and showed similar increases in response to **CpG**-ODN.

DETD . . . C57BL/6 spleen cells were cultured in two ml RPMI (supplemented as described for Example 1) with or without 40 μM **CpG** or non-**CpG** ODN for forty-eight hours. Cells were washed, and then used as effector cells in a short term ^{51} Cr release. . .

DETD In vivo Studies with **CpG** Phosphorothioate ODN

DETD B cells were cultured with phosphorothioate ODN with the sequence of control ODN 1a or the **CpG** ODN 1d and 3Db and then either pulsed after 20 hr with ^{3} H uridine or after 44 hr with. . .

DETD . . . for 1 hr. at 37 C. in the presence or absence of LPS or the control ODN 1a or the **CpG** ODN 1d and 3Db before addition of anti-IgM (1 μ/ml). Cells were cultured for a further 20 hr. before a. . .

DETD DBA/2 female mice (2 mos. old) were injected IP with 500 g **CpG** or control phosphorothioate ODN. At various time points after injection, the mice were bled. Two mice were studied for each. . .

DETD . . . (2U/μg of DNA) at 37° C. for 2 hr in 1×SSC with 5 mM MgCl2. To methylate the cytosine in **CpG** dinucleotides in E. coli DNA, E. coli DNA was treated with **CpG** methylase (M. SssI; 2 U/μg of DNA) in NEBuffer 2 supplemented with 160 μM S-adenosyl methionine and incubated overnight at. . .

DETD . . . humidified incubator maintained in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 1.5 mM L-glutamine, 50 μg/ml), **CpG** or non-**CpG** phosphodiester ODN (O-ODN) (20 μM), phosphorothioate ODN (S-ODN) (0.5 μM), or E. coli or calf thymus DNA (50 μg/ml) at. . . IgM production). Concentrations of stimulants were chosen based on preliminary studies with titrations. In some cases, cells were treated with **CpG** O-ODN along with various concentrations (1-10 μg/ml) of neutralizing rat IgG1 antibody against murine IL-6 (hybridoma MP5-20F3) or control rat. . .

DETD . . . injected intravenously (iv) with PBS, calf thymus DNA (200 μg/100 μl PBS/mouse), E. coli DNA (200 μg/100 μl PBS/mouse), or **CpG** or non-**CpG** S-ODN (200 μg/100 μl PBS/mouse). Mice (two/group) were bled by retroorbital puncture and sacrificed by cervical dislocation at various time. . .

DETD Cell Proliferation assay. DBA/2 mice spleen B cells (5×10^{4} cells/100 μl/well) were treated with media, **CpG** or non-**CpG** S-ODN (0.5 μM) or O-ODN (20 μM) for 24 hr at 37° C. Cells were pulsed for the last four. . .

DETD . . . a receptor-dependent mechanism. J. Clin. Invest. 93:944) at 250 mV and 960 μF. Cells were stimulated with various concentrations or **CpG** or non-**CpG** ODN after electroporation. Chloramphenicol acetyltransferase (CAT) activity was measured by a solution assay (Seed, B. and J. Y. Sheen (1988). . .

DETD Oligodeoxynucleotide Modifications Determine the Magnitude of B Cell Stimulation by **CpG** Motifs

DETD . . . central linkages are phosphodiester, but the two 5' and five 3' linkages are methylphosphonate modified. The ODN sequences studied (with **CpG** dinucleotides indicated by underlining) include:

DETD These sequences are representative of literally hundreds of **CpG** and non-**CpG** ODN that have been tested in the course of these studies.

DETD . . . (1990) J. Immunol 145:1039; and Ballas, Z. K. and W. Rasmussen (1993) J. Immunol, 150:17), with medium alone or with **CpG** or non-**CpG** ODN at the indicated concentrations, or with E.coli or calf thymus (50 μg/ml) at 37° C. for 24 hr. All. . .

DETD . . . immunized mice were then treated with oligonucleotides (30 μg in 200 μl saline by i.p.injection), which either contained an unmethylated **CpG** motif (i.e., **TCCATGACGTTCCTGACGTT**; SEQ ID NO.10) or did not (i.e., control, TCCATGAGCTTCCTGAGTCT; SEQ ID NO.8). Soluble SEA (10 μg in 25 μl of. . .

DETD . . . inflammatory cells are present in the lungs. However, when the mice are initially given a nucleic acid containing an unmethylated **CpG** motif along with the eggs, the inflammatory cells in the lung are not

increased by subsequent inhalation of the egg. . .

DETD . . . inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a **CpG** oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes. . .

DETD FIG. 14 shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can actually redirect the cytokine response of the lung to production of Il-12, indicating a Th1 type of immune. . .

DETD FIG. 15 shows that administration of an oligonucleotide containing an unmethylated **CpG** motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune. .

.

DETD **CpG** Oligonucleotides Induce Human PBMC to Secrete Cytokines

DETD . . . standard centrifugation over ficoll hypaque. Cells (5×10^5 /ml) were cultured in 10% autologous serum in 96 well microtiter plates with **CpG** or control oligodeoxynucleotides (24 μg/ml for phosphodiester oligonucleotides; 6 μ/ml for nuclease resistant phosphorothioate oligonucleotides) for 4 hr in the. . .

. . . Il-6 in a subject comprising administering to the subject an effective amount to induce Il-6 in the subject of an **immunostimulatory** nucleic acid, having a sequence comprising: 5'X_1 X_2 CGX_3 X3' wherein C is unmethylated, wherein X_1, X_2 and X_3, X_4. . .

. . . 1, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, GpT, GpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

11. The method of claim 1, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

12. The method of claim 1, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and. .

.

13. The method of claim 1, wherein the **immunostimulatory** nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCCATGTCGCTCCTGATGCT (SEQ ID NO:37); TCCATAACGTTCCTGATGCT. . .

14. A method of stimulating natural killer cell lytic activity comprising exposing a natural killer cell to an **immunostimulatory** nucleic acid to stimulate natural killer cell lytic activity, the **immunostimulatory** nucleic acid having a sequence comprising: 5'X_1 X_2 CGX_3 X3' wherein C is unmethylated, wherein X_1 X_2 and X_3 X_4. . .

. . . 14, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

23. The method of claim 14, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

24. The method of claim 15, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and.

. .

25. The method of claim 14, wherein the **immunostimulatory** nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCGTCGTTGTCGTTGTCGTT (SEQ ID NO:47); TCCATGACGGTCCTGATGCT. . .

. . . to a subject having an immune system deficiency an effective amount to induce interferon-gamma production in the subject of an **immunostimulatory** nucleic acid, having a sequence comprising: 5'X_1 X_2 CGX_3 X_4 3' wherein C is unmethylated, wherein X_1 X_2 and X_3. . .

. . . 26, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

35. The method of claim 26, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

36. The method of claim 26, wherein the **immunostimulatory** nucleic

acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and. . .

. . . Il-12 in a subject comprising: administering to the subject an effective amount to induce Il-12 in the subject, of an **immunostimulatory** nucleic acid having a sequence comprising: 5'X_1 X_2 CGX_3 X3' wherein C is unmethylated, wherein X_1, X_2, X_3, and X_4. . .

39. The method of claim 37, wherein the **immunostimulatory** nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCCATGTCGCTCCTGATGCT (SEQ ID NO:37); TCCATGACGATCCTGATGCT. . .

. . . 37, wherein X_1 X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, **CpG**, TpA, TpT, and TpG; and X_3 X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, **CpG**, TpC, ApC, CpC, TpA, ApA, and CpA.

48. The method of claim 37, wherein the **immunostimulatory** nucleic acid is 8 to 40 nucleotides in length.

49. The method of claim 37, wherein the **immunostimulatory** nucleic acid, has a sequence comprising: 5'NX_1 X_2 CGX_3 X_4 N3' wherein X_1, X_2, X_3, and X_4 are nucleotides and. . .

```
=> d his

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     FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006
                E GARCON NATALIE/IN
L1            25 S E4-E7
L2             8 S L1 AND (CPG)
L3            17 S L1 NOT L2
                E FRIEDE MARTIN/IN
L4            17 S E3
L5            12 S L4 NOT L1
L6            12 S (WD1001 OR WD1002 OR WD1003 OR WD1004 OR WD1005 OR WD1006 OR
L7            11 S L6 NOT L1
L8            11 S L7 NOT L4
L9             1 S L8 AND (CPG)
L10          154 S (TCCATGACGTTCCTGACGTT)
L11          154 S L10 NOT L1
L12          154 S L11 NOT L4
L13          154 S L12 AND (CPG)
L14          138 S L13 AND (IMMUNOSTIMULATORY)
L15           12 S L14 AND AY<2000
L16          110 S (TCTCCCAGCGTGCGCCAT)
L17            8 S L16 AND L15

=> s (ACCGATAACGTTGCCGGTGACG)
L18            1 (ACCGATAACGTTGCCGGTGACG)

=> d l18,cbib

L18  ANSWER 1 OF 1  USPATFULL on STN
2006:203076 Modification of MYD88 splicing using modified oligonucleotides.
    Vickers, Timothy, Oceanside, CA, UNITED STATES
    Dean, Nicholas M., Olivehain, CA, UNITED STATES
    US 2006172962 A1 20060803
    APPLICATION: US 2006-339785  A1  20060124 (11)
    PRIORITY: US 2005-648823P  20050131 (60)
    DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.


=> s (TCCATGAGCTTCCTGAGCTT)
L19            9 (TCCATGAGCTTCCTGAGCTT)

=> s l19 and l16
L20            8 L19 AND L16

=> s l20 and ay<2000
       3009073 AY<2000
L21            0 L20 AND AY<2000
```

=> d 120,cbib,8

L20 ANSWER 8 OF 8 USPATFULL on STN
2003:37157 Methods for enhancing antibody-induced cell lysis and treating cancer.
Weiner, George, Iowa City, IA, UNITED STATES
Hartmann, Gunther, Munich, GERMANY, FEDERAL REPUBLIC OF
US 2003026801 A1 20030206
APPLICATION: US 2001-888326 A1 20010622 (9)
PRIORITY: US 2000-213346P 20000622 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

=> d 120,cbib,1-8

L20 ANSWER 1 OF 8 USPATFULL on STN
2006:182486 Immunostimulatory nucleic acids for the treatment of asthma and allergy.
Bratzler, Robert L., Concord, MA, UNITED STATES
Petersen, Deanna M., Newton, MA, UNITED STATES
Fouron, Yves, Marlborough, MA, UNITED STATES
Coley Pharmaceutical Group, Inc., Wellesley, MA, UNITED STATES (U.S. corporation)
US 2006154890 A1 20060713
APPLICATION: US 2005-301360 A1 20051209 (11)
PRIORITY: US 2000-179991P 20000203 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L20 ANSWER 2 OF 8 USPATFULL on STN
2005:287460 Immunostimulatory nucleic acid for treatment of non-allergic inflammatory diseases.
Krieg, Arthur M., Wellesley, MA, UNITED STATES
Berg, Daniel J., Iowa City, IA, UNITED STATES
University of Iowa Research Foundation, Iowa City, IA, UNITED STATES (U.S. corporation)
US 2005250726 A1 20051110
APPLICATION: US 2005-127654 A1 20050512 (11)
PRIORITY: US 2001-279642P 20010329 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L20 ANSWER 3 OF 8 USPATFULL on STN
2004:299905 Immunostimulatory nucleic acids for the treatment of asthma and allergy.
Bratzler, Robert L., Concord, MA, UNITED STATES
Petersen, Deanna M., Newton, MA, UNITED STATES
Fouron, Yves, Marlboro, MA, UNITED STATES
US 2004235774 A1 20041125
APPLICATION: US 2004-831778 A1 20040423 (10)
PRIORITY: US 2000-179991P 20000203 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L20 ANSWER 4 OF 8 USPATFULL on STN
2003:300800 Immunostimulatory nucleic acids.
Krieg, Arthur M., Wellesley, MA, UNITED STATES
Schetter, Christian, Hilden, GERMANY, FEDERAL REPUBLIC OF
Vollmer, Jorg, Dusseldorf, GERMANY, FEDERAL REPUBLIC OF
University of Iowa Research Foundation, Iowa City, IA, 52242 (U.S. corporation)
US 2003212026 A1 20031113
APPLICATION: US 2002-314578 A1 20021209 (10)
PRIORITY: US 1999-156113P 19990925 (60)
US 1999-156135P 19990927 (60)
US 2000-227436P 20000823 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L20 ANSWER 5 OF 8 USPATFULL on STN
2003:127633 Immunostimulatory nucleic acids for the treatment of asthma and allergy.
Bratzler, Robert L., Concord, MA, UNITED STATES
Petersen, Deanna M., Newton, MA, UNITED STATES
Fouron, Yves, Marlboro, MA, UNITED STATES
US 2003087848 A1 20030508
APPLICATION: US 2001-776479 A1 20010202 (9)
PRIORITY: US 2000-179991P 20000203 (60)

DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L20 ANSWER 6 OF 8 USPATFULL on STN
2003:79087 Inhibition of angiogenesis by nucleic acids.
Bratzler, Robert L., Concord, MA, UNITED STATES
US 2003055014 A1 20030320
APPLICATION: US 2001-17995 A1 20011214 (10)
PRIORITY: US 2000-255534P 20001214 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L20 ANSWER 7 OF 8 USPATFULL on STN
2003:71981 Immunostimulatory nucleic acid for treatment of non-allergic inflammatory diseases.
Krieg, Arthur M., Wellesley, MA, UNITED STATES
Berg, Daniel J., Iowa City, IA, UNITED STATES
US 2003050268 A1 20030313
APPLICATION: US 2002-112653 A1 20020329 (10)
PRIORITY: US 2001-279642P 20010329 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L20 ANSWER 8 OF 8 USPATFULL on STN
2003:37157 Methods for enhancing antibody-induced cell lysis and treating cancer.
Weiner, George, Iowa City, IA, UNITED STATES
Hartmann, Gunther, Munich, GERMANY, FEDERAL REPUBLIC OF
US 2003026801 A1 20030206
APPLICATION: US 2001-888326 A1 20010622 (9)
PRIORITY: US 2000-213346P 20000622 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

=> s (ACCGATGACGTCGCCGGTGACGGCACCACG)
L22 5 (ACCGATGACGTCGCCGGTGACGGCACCACG)

=> d l22,ti,1-5

L22 ANSWER 1 OF 5 USPATFULL on STN
TI Pharmaceutical compositions comprising a polynucleotide and optionally an antigen especially for vaccination

L22 ANSWER 2 OF 5 USPATFULL on STN
TI Methods related to immunostimulatory nucleic acid-induced interferon

L22 ANSWER 3 OF 5 USPATFULL on STN
TI Pharmaceutical composition comprising a polynucleotide and optionally an antigen especially for vaccination

L22 ANSWER 4 OF 5 USPATFULL on STN
TI Methods for treating cancer

L22 ANSWER 5 OF 5 USPATFULL on STN
TI Dendritic cells; methods

=> d his

(FILE 'HOME' ENTERED AT 00:18:42 ON 13 NOV 2006)

FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006
E GARCON NATALIE/IN
L1 25 S E4-E7
L2 8 S L1 AND (CPG)
L3 17 S L1 NOT L2
E FRIEDE MARTIN/IN
L4 17 S E3
L5 12 S L4 NOT L1
L6 12 S (WD1001 OR WD1002 OR WD1003 OR WD1004 OR WD1005 OR WD1006 OR
L7 11 S L6 NOT L1
L8 11 S L7 NOT L4
L9 1 S L8 AND (CPG)
L10 154 S (TCCATGACGTTCCTGACGTT)
L11 154 S L10 NOT L1
L12 154 S L11 NOT L4
L13 154 S L12 AND (CPG)
L14 138 S L13 AND (IMMUNOSTIMULATORY)

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L15          12 S L14 AND AY<2000
L16         110 S (TCTCCCAGCGTGCGCCAT)
L17           8 S L16 AND L15
L18           1 S (ACCGATAACGTTGCCGGTGACG)
L19           9 S (TCCATGAGCTTCCTGAGCTT)
L20           8 S L19 AND L16
L21           0 S L20 AND AY<2000
L22           5 S (ACCGATGACGTCGCCGGTGACGGCACCACG)

=> s l22 and l15
L23           0 L22 AND L15

=> d l22,cbib,1-5

L22  ANSWER 1 OF 5  USPATFULL on STN
2006:43268 Pharmaceutical compositions comprising a polynucleotide and
    optionally an antigen especially for vaccination.
    Wagner, Hermann, Eching, GERMANY, FEDERAL REPUBLIC OF
    Lipford, Grayson, Munich, GERMANY, FEDERAL REPUBLIC OF
    Heeg, Klaus, Marburg-Michelbach, GERMANY, FEDERAL REPUBLIC OF
    Coley Pharmaceutical GmbH, Langenfeld, GERMANY, FEDERAL REPUBLIC OF
    (non-U.S. corporation)
    US 7001890 B1 20060221
    WO 9832462  19980730
    APPLICATION: US 1999-355254  19980123 (9)
    WO 1998-EP367 19980123 20000222  PCT 371 date
    PRIORITY: EP 1997-101019  19970123
    DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L22  ANSWER 2 OF 5  USPATFULL on STN
2005:243067 Methods related to immunostimulatory nucleic acid-induced
    interferon.
    Hartmann, Gunther, Munich, GERMANY, FEDERAL REPUBLIC OF
    Bratzler, Robert L., Concord, MA, UNITED STATES
    Krieg, Arthur M., Iowa City, IA, UNITED STATES
    Coley Pharmaceutical Group, Inc., Wellesley, MA, UNITED STATES (U.S.
    corporation)University of Iowa Research Foundation, Iowa City, IA, UNITED
    STATES (U.S. corporation)Coley Pharmaceutical GmbH, Langenfeld, GERMANY,
    FEDERAL REPUBLIC OF (non-U.S. corporation)
    US 6949520 B1 20050927
    APPLICATION: US 2000-672126  20000927 (9)
    PRIORITY: US 1999-156147P  19990927 (60)
    DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L22  ANSWER 3 OF 5  USPATFULL on STN
2005:63560 Pharmaceutical composition comprising a polynucleotide and
    optionally an antigen especially for vaccination.
    Wagner, Hermann, Eching, GERMANY, FEDERAL REPUBLIC OF
    Lipford, Grayson, Watertown, MA, UNITED STATES
    Heeg, Klaus, Marburg-Michelbach, GERMANY, FEDERAL REPUBLIC OF
    Coley Pharmaceutical GmbH, Langenfeld, GERMANY, FEDERAL REPUBLIC OF
    (non-U.S. corporation)
    US 2005054601 A1 20050310
    APPLICATION: US 2004-894655  A1  20040716 (10)
    PRIORITY: EP 1997-101019  19970123
    DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L22  ANSWER 4 OF 5  USPATFULL on STN
2003:200430 Methods for treating cancer.
    Vicari, Alain, La Tour de Salvagny, FRANCE
    Caux, Christophe, Bressolles, FRANCE
    Schering Corporation (non-U.S. corporation)
    US 2003138413 A1 20030724
    APPLICATION: US 2002-304616  A1  20021126 (10)
    PRIORITY: US 2001-333434P  20011127 (60)
    DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L22  ANSWER 5 OF 5  USPATFULL on STN
2003:3544 Dendritic cells; methods.
    Kadowaki, Norimitsu, Kyoto, JAPAN
    Liu, Yong-Jun, Palo Alto, CA, UNITED STATES
    US 2003003579 A1 20030102
    APPLICATION: US 2001-11635  A1  20011022 (10)
    PRIORITY: US 2000-243232P  20001024 (60)
    DOCUMENT TYPE: Utility; APPLICATION.
```

=> d 122,cbib,clm,kwic,3

L22 ANSWER 3 OF 5 USPATFULL on STN
2005:63560 Pharmaceutical composition comprising a polynucleotide and optionally an antigen especially for vaccination.
Wagner, Hermann, Eching, GERMANY, FEDERAL REPUBLIC OF
Lipford, Grayson, Watertown, MA, UNITED STATES
Heeg, Klaus, Marburg-Michelbach, GERMANY, FEDERAL REPUBLIC OF
Coley Pharmaceutical GmbH, Langenfeld, GERMANY, FEDERAL REPUBLIC OF (non-U.S. corporation)
US 2005054601 A1 20050310
APPLICATION: US 2004-894655 A1 20040716 (10)
PRIORITY: EP 1997-101019 19970123
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
CLM What is claimed is:
1-23. (Canceled).

24. An immunomodulatory composition, comprising (a) an oligonucleotide 5-7 nucleotides long comprising a sequence of a binding site for a transcription factor or a part thereof, and (b) a pharmaceutically acceptable carrier chosen from liposomes and emulsions.

25. The composition of claim 24, wherein the oligonucleotide is seven nucleotides long.

26. The composition of claim 24, wherein the oligonucleotide is six nucleotides long.

27. The composition of claim 24, wherein the oligonucleotide is five nucleotides long.

28. The composition of claim 24, wherein the oligonucleotide comprises a sequence 5'-Pu-Pu-CpG-Py-Py-3', wherein Pu means purine and is chosen from adenine (A) and guanine (G) and wherein Py means pyrimidine and is chosen from cytosine (C), thymine (T), and uracil.

29. The composition of claim 24, wherein the oligonucleotide comprises a sequence 5'-Pu-Pu-CpG-Py-C-3', wherein Pu means purine and is chosen from adenine (A) and guanine (G) and wherein Py means pyrimidine and is chosen from cytosine (C), thymine (T), and uracil.

30. The composition of claim 24, wherein the oligonucleotide comprises a sequence GACGTC.

31. The composition of claim 24, wherein the oligonucleotide comprises at least one phosphorothioate linkage.

32. The composition of claim 24, further comprising an antigen.

33. The composition of claim 24, wherein said composition does not comprise an antigen.

34. A method of modulating an immune response in a patient, comprising administering to a patient a composition of any one of claims 24-32 in a suitable dose to modulate an immune response in the patient.

35. The method of claim 34, wherein the immune response is an immune response of the acquired immune system.

36. The method of claim 34, wherein the immune response is an immune response of the innate immune system.

37. The method of claim 34, wherein the suitable dose to modulate the immune response in the patient shifts an immune response to a Th1-type reactivity.

38. The method of claim 34, wherein the immune response is IgE-mediated allergy.

39. The method of claim 34, wherein the patient is receiving a vaccine.

40. The method of claim 39, wherein the vaccine comprises an antigen chosen from viral, bacterial, parasitic, transplantation, and tumor antigens.

41. The method of claim 34, wherein the patient suffers from cancer.

42. The method of claim 34, wherein the patient suffers from an infection.

43. A method of treating a patient suffering from an infection, comprising administering to a patient suffering from an infection a suitable dose of a composition of any one of claims 24-32 to treat the patient.

44. The method of claim 43, wherein the infection is chosen from Leishmaniasis, Toxoplasmosis, and Mycobacteriosis.

DETD . . . of E. coli, here termed AMP (TCATTGGAAAACGTTCTTCGGGGC). The second sequence is derived from a BCG gene and is termed BCG-A4A (**ACCGATGACGTCGCCGGTGACGGCACCACG**). The third is a synthetic sequence claimed to be a prototype of bacterial CpG sequences, referred to by Krieg et.al.. . .

```
=> d his

     (FILE 'HOME' ENTERED AT 00:18:42 ON 13 NOV 2006)

     FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006
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L1             25 S E4-E7
L2              8 S L1 AND (CPG)
L3             17 S L1 NOT L2
                 E FRIEDE MARTIN/IN
L4             17 S E3
L5             12 S L4 NOT L1
L6             12 S (WD1001 OR WD1002 OR WD1003 OR WD1004 OR WD1005 OR WD1006 OR
L7             11 S L6 NOT L1
L8             11 S L7 NOT L4
L9              1 S L8 AND (CPG)
L10           154 S (TCCATGACGTTCCTGACGTT)
L11           154 S L10 NOT L1
L12           154 S L11 NOT L4
L13           154 S L12 AND (CPG)
L14           138 S L13 AND (IMMUNOSTIMULATORY).
L15            12 S L14 AND AY<2000
L16           110 S (TCTCCCAGCGTGCGCCAT)
L17             8 S L16 AND L15
L18             1 S (ACCGATAACGTTGCCGGTGACG)
L19             9 S (TCCATGAGCTTCCTGAGCTT)
L20             8 S L19 AND L15
L21             0 S L20 AND AY<2000
L22             1 S (ACCGATGACGTCGCCGGTGACGGCACCACG)
L23             0 S L22 AND L15

=> s (HIV or human immunodeficiency virus)
         46090 HIV
        529468 HUMAN
         26015 IMMUNODEFICIENCY
        107288 VIRUS
         18524 HUMAN IMMUNODEFICIENCY VIRUS
                 (HUMAN(W)IMMUNODEFICIENCY(W)VIRUS)
L24      48505 (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)

=> s l24 and (gp120/clm or gp160/clm or Env/clm)
           545 GP120/CLM
           202 GP160/CLM
           656 ENV/CLM
L25       1086 L24 AND (GP120/CLM OR GP160/CLM OR ENV/CLM)

=> s l25 and (ALUM/clm or saponin/clm)
          1043 ALUM/CLM
           633 SAPONIN/CLM
L26         14 L25 AND (ALUM/CLM OR SAPONIN/CLM)

=> s l26 and (ALUM/clm and saponin/clm)
          1043 ALUM/CLM
           633 SAPONIN/CLM
L27          3 L26 AND (ALUM/CLM AND SAPONIN/CLM)

=> d l27,cbib,1-3

L27  ANSWER 1 OF 3  USPATFULL on STN
2006:60208 Particle-bound human immunodeficiency virus envelope
```

glycoproteins and related compositions and methods.
Olson, William C., Ossining, NY, UNITED STATES
Schulke, Norbert, New City, NY, UNITED STATES
Gardner, Jason, Ardsley, NY, UNITED STATES
Maddon, Paul J., Scarsdale, NY, UNITED STATES
US 2006051373 A1 20060309
APPLICATION: US 2002-510268 A1 20020906 (10)
WO 2002-US28332 20020906 20050711 PCT 371 date
PRIORITY: US 2002-370410P 20020405 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L27 ANSWER 2 OF 3 USPATFULL on STN
2005:104593 **Human immunodeficiency virus** envelope clycoprotein mutants and uses thereof.
Moore, John P., New York, NY, UNITED STATES
Binley, James M., San Diego, CA, UNITED STATES
Lu, Min, New York, NY, UNITED STATES
Olson, William C., New York, NY, UNITED STATES
Schulke, Norbert, New City, NY, UNITED STATES
Gardner, Jason, Ardsley, NY, UNITED STATES
Maddon, Paul J., Scarsdale, NY, UNITED STATES
Sanders, Rogier, Amsterdam, NETHERLANDS
US 2005089526 A1 20050428
APPLICATION: US 2003-489040 A1 20020906 (10)
WO 2002-US28331 20020906
PRIORITY: US 2001-317909P 20010906 (60)
US 2003-317764P 20010906 (60)
US 2003-317910P 20010906 (60)
US 2003-317775P 20010906 (60)
US 2003-370264P 20020405 (60)
US 2003-370410P 20020405 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L27 ANSWER 3 OF 3 USPATFULL on STN
2005:36964 Materials and methods for immunizing against FIV infection.
Yamamoto, Janet K., Gainesville, FL, UNITED STATES
US 2005031639 A1 20050210
APPLICATION: US 2004-844658 A1 20040512 (10)
PRIORITY: US 2003-470066P 20030512 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

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E GARCON NATALIE/IN
L1 25 S E4-E7
L2 8 S L1 AND (CPG)
L3 17 S L1 NOT L2
E FRIEDE MARTIN/IN
L4 17 S E3
L5 12 S L4 NOT L1
L6 12 S (WD1001 OR WD1002 OR WD1003 OR WD1004 OR WD1005 OR WD1006 OR
L7 11 S L6 NOT L1
L8 11 S L7 NOT L4
L9 1 S L8 AND (CPG)
L10 154 S (TCCATGACGTTCCTGACGTT)
L11 154 S L10 NOT L1
L12 154 S L11 NOT L4
L13 154 S L12 AND (CPG)
L14 138 S L13 AND (IMMUNOSTIMULATORY)
L15 12 S L14 AND AY<2000
L16 110 S (TCTCCCAGCGTGCGCCAT)
L17 8 S L16 AND L15
L18 1 S (ACCGATAACGTTGCCGGTGACG)
L19 9 S (TCCATGAGCTTCCTGAGCTT)
L20 8 S L19 AND L16
L21 0 S L20 AND AY<2000
L22 5 S (ACCGATGACGTCGCCGGTGACGGCACCACG)
L23 0 S L22 AND L15
L24 48505 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L25 1086 S L24 AND (GP120/CLM OR GP160/CLM OR ENV/CLM)
L26 14 S L25 AND (ALUM/CLM OR SAPONIN/CLM)
L27 3 S L26 AND (ALUM/CLM AND SAPONIN/CLM)

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       3009073 AY<2000
L29          1 L28 AND AY<2000

=> d l29,cbib

L29  ANSWER 1 OF 1  USPATFULL on STN
2002:216828 Vaccines against cancer and infectious diseases.
    Goldenberg, David M., Short Hills, NJ, United States
    Hansen, Hans J., Westfield, NJ, United States
    Immunomedics, Inc., Morris Plains, NJ, United States (U.S. corporation)
    US 6440416 B1 20020827
    APPLICATION: US 1992-183381  19920102 (8)
    DOCUMENT TYPE: Utility; GRANTED.


=> d l29,cbib,clm,kwic

L29  ANSWER 1 OF 1  USPATFULL on STN
2002:216828 Vaccines against cancer and infectious diseases.
    Goldenberg, David M., Short Hills, NJ, United States
    Hansen, Hans J., Westfield, NJ, United States
    Immunomedics, Inc., Morris Plains, NJ, United States (U.S. corporation)
    US 6440416 B1 20020827
    APPLICATION: US 1992-183381  19920102 (8)
    DOCUMENT TYPE: Utility; GRANTED.
CLM    What is claimed is:
       1. A method of stimulating an immune response in a human against
       malignant cells or an infectious agent, which comprises the step of
       administering to said human an immunogenic amount of a baboon
       anti-idiotype antibody or antibody fragment that acts as an immunogenic
       functional mimic of an antigen that is a marker for a malignant cell or
       an infectious agent.

       2. The method of claim 1, wherein said baboon anti-idiotype antibody or
       antibody fragment is an immunogenic functional mimic of an epitope on
       said antigen.

       3. The method of claim 1, wherein said antigen is a marker for a
       malignant cell.

       4. The method of claim 3, wherein said antigen is carcinoembryonic
       antigen.

       5. The method of claim 4, wherein said baboon anti-idiotype antibody or
       antibody fragment acts as an immunogenic mimic of an epitope on
       carcinoembryonic antigen which is not shared with either nonspecific
       crossreacting antigen or meconium antigen.

       6. The method of claim 1, wherein said antigen is a marker for a virus.

       7. The method of claim 6, wherein said antigen is a human
       immunodeficiency virus envelope protein.

       8. The method of claim 7, wherein said envelope protein is gp120.

       9. The method of claim 1, wherein said antigen is a marker for an
       infectious microorganism selected from the group consisting of bacteria,
       rickettsia, mycoplasma, protozoa and fungi.

       10. The method of claim 1, wherein said antigen is a marker for an
       infectious parasite.

       11. The method of claim 1, wherein said baboon anti-idiotype antibody or
       antibody fragment is administered in combination with an immunostimulant
       adjuvant.

       12. The method of claim 11, wherein said adjuvant is at least one member
       selected from the group consisting of Freund's adjuvant, alum,
       Bacillus Calmette-Guerin and tetanus toxoid.

       13. The method of claim 1, further comprising the administration of said
       antigen.

       14. The method of claim 1, wherein said human is a cancer patient
```

suffering from a malignant solid tumor or hematopoietic neoplasm.

15. The method of claim 14, wherein said malignant solid tumor or hematopoietic neoplasm is a gastrointestinal, lung, breast, prostate, ovarian, testicular, brain or lymphatic lesion, a sarcoma or a melanoma lesion.

16. The method of claim 1, wherein said human is suffering from a viral infection.

17. The method of claim 1, wherein said human is suffering from infection by an infectious microorganism selected from the group consisting of bacteria, rickettsia, mycoplasma, protozoa and fungi.

18. The method of claim 1, wherein said human is suffering from infection by an infectious parasite.

19. The method of claim 1, wherein said human is not suffering from a malignancy or from an infection, and said immune response results in immunity against the development of malignancy by a cell having said malignant cell marker, or against infection by an infectious agent having said infectious agent marker.

20. An antitumor or antipathogen vaccine, comprising an immunogenic amount of a baboon anti-idiotype antibody or antibody fragment that is an immunogenic functional mimic of an antigen that is a marker for a malignant cell or infectious agent, and a physiologically acceptable vaccine vehicle.

21. The vaccine of claim 20, wherein said vehicle comprises an effective amount of an immunostimulant adjuvant.

22. The vaccine of claim 21, wherein said adjuvant is at least one member selected from the group consisting of Freund's adjuvant, **alum**, Bacillus Calmette-Guerin and tetanus toxoid.

23. The vaccine of claim 20, which further comprises said antigen.

24. The vaccine of claim 22, which further comprises said antigen.

25. The method of claim 1, wherein said antigen is carcinoembryonic antigen.

26. The method of claim 1, wherein said baboon anti-idiotype antibody or antibody fragment acts as an immunogenic mimic of an epitope on carcinoembryonic antigen which is not shared with either nonspecific cross-reacting antigen or meconium antigen.

AI **US 1992-183381 19920102 (8)**

SUMM MAbs against the gp120 glycoprotein antigen of **human immunodeficiency virus** 1 (**HIV**-1) are known, and certain of such antibodies can have an immunoprotective role in humans. See, e.g., Rossi et al., Proc.. . .

SUMM. . . . tumor antigen preparation, isolated tumor antigens and/or oligopeptide fragments thereof, or viral coat proteins and/or fragments thereof (such as the **HIV**-1 gp-120 peptide), microbial cell membrane or cell wall components, parasite surface antigens, portions thereof, or fragments resulting from destruction of. . .

SUMM . . . CEA-producing cancers. A description is also provided for production of anti-idiotype antibodies mimicking the gp-120 viral coat glycoprotein component of **human immunodeficiency virus HIV**-1, implicated in AIDS, and use thereof to confer immunity against infection by the AIDS virus.

DETD Baboon Anti-**HIV**-1 Ab2 Antibody Preparation

DETD Pristane-primed Balb/c mice are repeatedly immunized with **human immunodeficiency virus** 1 (**HIV**-1) envelope glycoprotein gp120, in complete Freund's adjuvant. After several weeks, the mice are sacrificed, their spleens are excised, and spleen. . . cells with murine myeloma cells is effected and resultant hybridomas are selected and cloned for production and secretion of monoclonal anti-**HIV**-1 antibodies that specifically bind to gp120.

DETD The monoclonal anti-**HIV**-1 idiotype antibodies are used to immunize baboons to produce Ab2 antibodies, according to the procedure of Example 1. The antiserum. . .

DETD A test group of 20 male intravenous drug users who test negative for **HIV**-1 antibodies are divided into two paired subgroups. The members of the first subgroup are each immunized with the affinity purified. . .

DETD The members of each group are followed for three years and tested periodically for anti-**HIV** serum antibodies and development of ARC and

AIDS symptoms. After three years, seven members of the second group are seropositive,. . . suggesting that he is undetectably infected at the start of the trial. The remaining members of the first group develop anti-**HIV** antibodies within 620 weeks after the end of the immunization schedule, but do not develop AIDS symptoms during the three. . .
7. The method of claim 6, wherein said antigen is a **human immunodeficiency virus** envelope protein.

8. The method of claim 7, wherein said envelope protein is **gp120**.

12. The method of claim 11, wherein said adjuvant is at least one member selected from the group consisting of Freund's adjuvant, **alum**, Bacillus Calmette-Guerin and tetanus toxoid.

. . . vaccine of claim 21, wherein said adjuvant is at least one member selected from the group consisting of Freund's adjuvant, **alum**, Bacillus Calmette-Guerin and tetanus toxoid.

=> d

L29 ANSWER 1 OF 1 USPATFULL on STN
Full Text
AN 2002:216828 USPATFULL
TI Vaccines against cancer and infectious diseases
IN Goldenberg, David M., Short Hills, NJ, United States
Hansen, Hans J., Westfield, NJ, United States
PA Immunomedics, Inc., Morris Plains, NJ, United States (U.S. corporation)
PI US 6440416 B1 20020827
AI US 1992-183381 19920102 (8)
RLI Continuation of Ser. No. US 1990-470637, filed on 26 Jan 1990, now abandoned
DT Utility
FS GRANTED
LN.CNT 869
INCL INCLM: 424/131.100
INCLS: 424/138.100; 424/148.100; 424/160.100; 424/164.100; 424/151.100; 530/387.200; 530/387.700; 530/388.300; 530/388.200; 530/388.400; 530/388.500; 530/388.600
NCL NCLM: 424/131.100
NCLS: 424/138.100; 424/148.100; 424/151.100; 424/160.100; 424/164.100; 530/387.200; 530/387.700; 530/388.200; 530/388.300; 530/388.400; 530/388.500; 530/388.600
IC [7]
ICM A61K039-395
ICS A61K039-42; A61K039-40; C07K016-00
IPCI A61K0039-395 [ICM,7]; A61K0039-42 [ICS,7]; A61K0039-40 [ICS,7]; C07K0016-00 [ICS,7]
IPCR A61K0039-395 [I,A]; A61K0039-395 [I,C*]
EXF 424/131.1; 424/138.1; 424/148.1; 424/160.1; 424/164.1; 424/151.1; 530/387.2; 530/387.7; 530/388.3; 530/388.35; 530/388.5; 530/388.6; 530/388.2; 530/388.4

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FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006
E GARCON NATALIE/IN
L1 25 S E4-E7
L2 8 S L1 AND (CPG)
L3 17 S L1 NOT L2
E FRIEDE MARTIN/IN
L4 17 S E3
L5 12 S L4 NOT L1
L6 12 S (WD1001 OR WD1002 OR WD1003 OR WD1004 OR WD1005 OR WD1006 OR
L7 11 S L6 NOT L1
L8 11 S L7 NOT L4
L9 1 S L8 AND (CPG)
L10 154 S (TCCATGACGTTCCTGACGTT)
L11 154 S L10 NOT L1
L12 154 S L11 NOT L4
L13 154 S L12 AND (CPG)
L14 138 S L13 AND (IMMUNOSTIMULATORY)
L15 12 S L14 AND AY<2000
L16 110 S (TCTCCCAGCGTGCGCCAT)
L17 8 S L16 AND L15

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L18           1 S (ACCGATAACGTTGCCGGTGACG)
L19           9 S (TCCATGAGCTTCCTGAGCTT)
L20           8 S L19 AND L16
L21           0 S L20 AND AY<2000
L22           5 S (ACCGATGACGTCGCCGGTGACGGCACCACG)
L23           0 S L22 AND L15
L24       48505 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L25        1086 S L24 AND (GP120/CLM OR GP160/CLM OR ENV/CLM)
L26          14 S L25 AND (ALUM/CLM OR SAPONIN/CLM)
L27           3 S L26 AND (ALUM/CLM AND SAPONIN/CLM)
L28          11 S L26 NOT L27
L29           1 S L28 AND AY<2000

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COST IN U.S. DOLLARS                          SINCE FILE      TOTAL
                                                 ENTRY    SESSION
FULL ESTIMATED COST                             241.81     242.02

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MOST RECENT THOMSON SCIENTIFIC UPDATE:     200672      <200672/DW>
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PLEASE VISIT:
http://www.stn-international.de/training_center/patents/stn_guide.pdf

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http://scientific.thomson.com/support/patents/coverage/latestupdates/

PLEASE BE AWARE OF THE NEW IPC REFORM IN 2006, SEE
http://www.stn-international.de/stndatabases/details/ipc_reform.html and
http://scientific.thomson.com/media/scpdf/ipcrdwpi.pdf


>>> FOR DETAILS ON THE NEW AND ENHANCED DERWENT WORLD PATENTS INDEX
    PLEASE SEE
http://www.stn-international.de/stndatabases/details/dwpi_r.html <<<


>>> YOU ARE IN THE NEW AND ENHANCED DERWENT WORLD PATENTS INDEX <<<

=> e bruck c/in
E1            1     BRUCK B/IN
E2            1     BRUCK B S/IN
E3           13 --> BRUCK C/IN
E4           10     BRUCK C E M/IN
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E11           6     BRUCK D W/IN
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             10 "BRUCK C E M"/IN
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                K C T"/IN)

=> s l30 and (HIV)
          23487 HIV
L31           8 L30 AND (HIV)

=> s l31 and (Nef)
            404 NEF
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L32 2 L31 AND (NEF)

=> d l32,ti,1-2

L32 ANSWER 1 OF 2 WPIDS COPYRIGHT 2006 THE THOMSON CORP on STN

TI Vaccine

L32 ANSWER 2 OF 2 WPIDS COPYRIGHT 2006 THE THOMSON CORP on STN

TI **HIV** Tat or **Nef** protein linked to a fusion partner

=> d l32,bib,ab,1-2

L32 ANSWER 1 OF 2 WPIDS COPYRIGHT 2006 THE THOMSON CORP on STN
Full Text
AN 2005-557652 [57] WPIDS
DNC C2005-168053 [57]
TI Vaccine
DC B04; D16
IN **BRUCK C**; GEORGES M M; GODART S A
PA (SMIK-C) SMITHKLINE BEECHAM BIOLOGICALS
CYC 1
PIA IN 9802172 I4 20050304 (200557)* EN [0]
ADT IN 9802172 I4 IN 1998-CH2172 19980925
PRAI EP 1997-205850 19970926
AB IN 9802172 I4 UPAB: 20051223
NOVELTY - The invention provides:(a) an **HIV** Tat protein or its derivative linked to either:(i) a fusion partner; or(ii) an **HIV Nef** protein or its derivative; or (b) an **HIV Nef** protein or its derivative linked to either:(i) a fusion partner; or (ii) an **HIV** Tat protein or its derivative; or (c) an **HIV Nef** protein or its derivative linked to an **HIV** Tat protein or its derivative and a fusion partner.The invention further provides for a nucleic acid encoding the protein and a host cell, such as Pichia Pastoris, transformed with the nucleic acid. Image 0/0

L32 ANSWER 2 OF 2 WPIDS COPYRIGHT 2006 THE THOMSON CORP on STN
Full Text
AN 1999-302282 [25] WPIDS
DNC C1999-088588 [25]
TI **HIV** Tat or **Nef** protein linked to a fusion partner
DC B04; D16
IN **BRUCK C**; GODART S A G; MARCHAND M
PA (SMIK-C) SMITHKLINE BEECHAM BIOLOGICALS; (GLAX-C) GLAXOSMITHKLINE BIOLOGICALS SA
CYC 83
PIA WO 9916884 A1 19990408 (199925)* EN 66
AU 9910255 A 19990423 (199935) EN
ZA 9808789 A 20000531 (200032) EN 73
EP 1015596 A1 20000705 (200035) EN
NO 2000001508 A 20000518 (200036) NO
BR 9812547 A 20000725 (200043) PT
CZ 2000001091 A3 20000913 (200054) CS
CN 1279718 A 20010110 (200128) ZH
HU 2000004896 A2 20010428 (200131) HU
KR 2001024287 A 20010326 (200161) KO
NZ 503482 A 20010928 (200161) EN
JP 2001518300 W 20011016 (200176) JA 73
AU 746564 B 20020502 (200238) EN
TW 499436 A 20020821 (200333) ZH
AU 2002300390 A1 20030206 (200427) EN
US 20050033022 A1 20050210 (200512) EN
CN 1188519 C 20050209 (200622) ZH
EP 1015596 B1 20060830 (200657) EN
DE 69835756 E 20061012 (200670) DE
ADT WO 9916884 A1 WO 1998-EP6040 19980917; BR 9812547 A BR 1998-12547 19980917; CN 1279718 A CN 1998-811432 19980917; CN 1188519 C CN 1998-811432 19980917; EP 1015596 A1 EP 1998-952625 19980917; EP 1015596 B1 EP 1998-952625 19980917; NZ 503482 A NZ 1998-503482 19980917; EP 1015596 A1 WO 1998-EP6040 19980917; NO 2000001508 A WO 1998-EP6040 19980917; BR 9812547 A WO 1998-EP6040 19980917; CZ 2000001091 A3 WO 1998-EP6040 19980917; HU 2000004896 A2 WO 1998-EP6040 19980917; NZ 503482 A WO 1998-EP6040 19980917; JP 2001518300 W WO 1998-EP6040 19980917; US 20050033022 A1 Cont of WO 1998-EP6040 19980917; EP 1015596 B1 WO 1998-EP6040 19980917; ZA 9808789 A ZA 1998-8789 19980925; TW 499436 A TW 1998-117493 19981022; AU 9910255 A AU 1999-10255 19980917; AU 746564 B AU 1999-10255 19980917; CZ 2000001091 A3 CZ 2000-1091 19980917; HU 2000004896 A2 HU 2000-4896 19980917; JP 2001518300 W JP 2000-513953 19980917; NO

2000001508 A NO 2000-1508 20000323; US 20050033022 A1 Cont of US 2000-509239 20000323; KR 2001024287 A KR 2000-703180 20000324; AU 2002300390 A1 AU 2002-300390 20020802; US 20050033022 A1 US 2003-687060 20031016; DE 69835756 E DE 1998-635756 19980917; DE 69835756 E EP 1998-952625 19980917; DE 69835756 E WO 1998-EP6040 19980917

FDT AU 746564 B Previous Publ AU 9910255 A; AU 2002300390 A1 Div ex AU 746564 B; AU 9910255 A Based on WO 9916884 A; EP 1015596 A1 Based on WO 9916884 A; BR 9812547 A Based on WO 9916884 A; CZ 2000001091 A3 Based on WO 9916884 A; HU 2000004896 A2 Based on WO 9916884 A; NZ 503482 A Based on WO 9916884 A; JP 2001518300 W Based on WO 9916884 A; AU 746564 B Based on WO 9916884 A; EP 1015596 B1 Based on WO 9916884 A; DE 69835756 E Based on EP 1015596 A; DE 69835756 E Based on WO 9916884 A

PRAI GB 1997-20585 19970926

AB WO 1999016884 A1 UPAB: 20050829

NOVELTY - A protein comprising a **HIV** Tat or **Nef** protein linked to a fusion partner.

DETAILED DESCRIPTION - A protein comprising:

(1) an **HIV** Tat protein or derivative linked to either a fusion partner or an **HIV Nef** protein or derivative; or

(2) an **HIV Nef** protein or derivative linked to either a fusion partner or an **HIV** Tat protein or derivative; or

(3) an **HIV Nef** protein or derivative linked to a **HIV** Tat protein or derivative and a fusion partner.

INDEPENDENT CLAIMS are also included for:

(1) a nucleic acid encoding the above protein;

(2) a host transformed with the nucleic acid of (1);

(3) a vaccine comprising the above protein;

(4) a method for producing the above protein, comprising transforming a host (preferably E. coli or Pichia pastoris) with a nucleic acid encoding the protein, expressing the protein and recovering the protein;

(5) a method for producing the vaccine of (3) comprising admixing the protein with a pharmaceutically acceptable diluent; and

(6) a method for preparing an **HIV Nef** protein or derivative and/or **HIV** Tat protein or derivative, in Pichia pastoris comprising transforming Pichia pastoris with DNA encoding the **HIV Nef** or Tat protein or derivative, expressing the protein and recovering the protein.

ACTIVITY - Antiviral; AntiHIV.

MECHANISM OF ACTION - Vaccine

USE - The protein can be used in a vaccine (claimed) to prevent **HIV** infection.

ADVANTAGE - None given.

=> d hi

(FILE 'HOME' ENTERED AT 00:18:42 ON 13 NOV 2006)

FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006

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L1           25 S E4-E7
L2            8 S L1 AND (CPG)
L3           17 S L1 NOT L2
                E FRIEDE MARTIN/IN
L4           17 S E3
L5           12 S L4 NOT L1
L6           12 S (WD1001 OR WD1002 OR WD1003 OR WD1004 OR WD1005 OR WD1006 OR
L7           11 S L6 NOT L1
L8           11 S L7 NOT L4
L9            1 S L8 AND (CPG)
L10         154 S (TCCATGACGTTCCTGACGTT)
L11         154 S L10 NOT L1
L12         154 S L11 NOT L4
L13         154 S L12 AND (CPG)
L14         138 S L13 AND (IMMUNOSTIMULATORY)
L15          12 S L14 AND AY<2000
L16         110 S (TCTCCCAGCGTGCGCCAT)
L17           8 S L16 AND L15
L18           1 S (ACCGATAACGTTGCCGGTGACG)
L19           9 S (TCCATGAGCTTCCTGAGCTT)
L20           8 S L19 AND L16
L21           0 S L20 AND AY<2000
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L23           0 S L22 AND L15
L24       48505 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L25        1086 S L24 AND (GP120/CLM OR GP160/CLM OR ENV/CLM)
L26          14 S L25 AND (ALUM/CLM OR SAPONIN/CLM)
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L27           3 S L26 AND (ALUM/CLM AND SAPONIN/CLM)
L28          11 S L26 NOT L27
L29           1 S L28 AND AY<2000

     FILE 'WPIDS' ENTERED AT 00:59:06 ON 13 NOV 2006
                E BRUCK C/IN
L30          22 S E3-E6
L31           8 S L30 AND (HIV)
L32           2 S L31 AND (NEF)

=> s l32 and (protein D)
        156512 PROTEIN
        613894 D
           256 PROTEIN D
                 (PROTEIN(W)D)
L33           1 L32 AND (PROTEIN D)

=> d l33,bib

L33  ANSWER 1 OF 1  WPIDS COPYRIGHT 2006       THE THOMSON CORP on STN
Full Text
AN   1999-302282 [25]   WPIDS
DNC  C1999-088588 [25]
TI   HIV Tat or Nef protein linked to a fusion partner
DC   B04; D16
IN   BRUCK C; GODART S A G; MARCHAND M
PA   (SMIK-C) SMITHKLINE BEECHAM BIOLOGICALS; (GLAX-C) GLAXOSMITHKLINE
     BIOLOGICALS SA
CYC  83
PIA  WO 9916884      A1 19990408 (199925)* EN  66
     AU 9910255      A  19990423 (199935)  EN
     ZA 9808789      A  20000531 (200032)  EN  73
     EP 1015596      A1 20000705 (200035)  EN
     NO 2000001508   A  20000518 (200036)  NO
     BR 9812547      A  20000725 (200043)  PT
     CZ 2000001091   A3 20000913 (200054)  CS
     CN 1279718      A  20010110 (200128)  ZH
     HU 2000004896   A2 20010428 (200131)  HU
     KR 2001024287   A  20010326 (200161)  KO
     NZ 503482       A  20010928 (200161)  EN
     JP 2001518300   W  20011016 (200176)  JA  73
     AU 746564       B  20020502 (200238)  EN
     TW 499436       A  20020821 (200333)  ZH
     AU 2002300390   A1 20030206 (200427)  EN
     US 20050033022  A1 20050210 (200512)  EN
     CN 1188519      C  20050209 (200622)  ZH
     EP 1015596      B1 20060830 (200657)  EN
     DE 69835756     E  20061012 (200670)  DE
ADT  WO 9916884 A1 WO 1998-EP6040 19980917; BR 9812547 A BR 1998-12547
     19980917; CN 1279718 A CN 1998-811432 19980917; CN 1188519 C CN
     1998-811432 19980917; EP 1015596 A1 EP 1998-952625 19980917; EP 1015596 B1
     EP 1998-952625 19980917; NZ 503482 A NZ 1998-503482 19980917; EP 1015596
     A1 WO 1998-EP6040 19980917; NO 2000001508 A WO 1998-EP6040 19980917; BR
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     19980917; HU 2000004896 A2 WO 1998-EP6040 19980917; NZ 503482 A WO
     1998-EP6040 19980917; JP 2001518300 W WO 1998-EP6040 19980917; US
     20050033022 A1 Cont of WO 1998-EP6040 19980917; EP 1015596 B1 WO
     1998-EP6040 19980917; ZA 9808789 A ZA 1998-8789 19980925; TW 499436 A TW
     1998-117493 19981022; AU 9910255 A AU 1999-10255 19980917; AU 746564 B AU
     1999-10255 19980917; CZ 2000001091 A3 CZ 2000-1091 19980917; HU 2000004896
     A2 HU 2000-4896 19980917; JP 2001518300 W JP 2000-513953 19980917; NO
     2000001508 A NO 2000-1508 20000323; US 20050033022 A1 Cont of US
     2000-509239 20000323; KR 2001024287 A KR 2000-703180 20000324; AU
     2002300390 A1 AU 2002-300390 20020802; US 20050033022 A1 US 2003-687060
     20031016; DE 69835756 E DE 1998-635756 19980917; DE 69835756 E EP
     1998-952625 19980917; DE 69835756 E WO 1998-EP6040 19980917
FDT  AU 746564       B Previous Publ AU 9910255      A; AU 2002300390   A1 Div
     ex AU 746564       B; AU 9910255      A Based on WO 9916884      A; EP
     1015596      A1 Based on WO 9916884      A; BR 9812547      A Based on WO
     9916884      A; CZ 2000001091   A3 Based on WO 9916884      A; HU
     2000004896   A2 Based on WO 9916884      A; NZ 503482       A Based on WO
     9916884      A; JP 2001518300   W Based on WO 9916884      A; AU 746564
     B Based on WO 9916884      A; EP 1015596      B1 Based on WO 9916884
     A; DE 69835756     E Based on EP 1015596      A; DE 69835756     E Based
     on WO 9916884      A
PRAI GB 1997-20585 19970926


=> file medline
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COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                       ENTRY    SESSION
FULL ESTIMATED COST                                    32.93     274.95

FILE 'MEDLINE' ENTERED AT 01:02:04 ON 13 NOV 2006

 FILE LAST UPDATED: 11 Nov 2006 (20061111/UP).  FILE COVERS 1950 TO DATE.

 On December 11, 2005, the 2006 MeSH terms were loaded.

 The MEDLINE reload for 2006 is now (26 Feb.) available.  For details
 on the 2006 reload, enter HELP RLOAD at an arrow prompt (=>).
 See also:

    http://www.nlm.nih.gov/mesh/
    http://www.nlm.nih.gov/pubs/techbull/nd04/nd04_mesh.html
    http://www.nlm.nih.gov/pubs/techbull/nd05/nd05_med_data_changes.html
    http://www.nlm.nih.gov/pubs/techbull/nd05/nd05_2006_MeSH.html

 OLDMEDLINE is covered back to 1950.

 MEDLINE thesauri in the /CN, /CT, and /MN fields incorporate the
 MeSH 2006 vocabulary.

 This file contains CAS Registry Numbers for easy and accurate
 substance identification.

=> s (HIV or human immunodeficiency virus)
        164536 HIV
       1430089 HUMAN
        125282 IMMUNODEFICIENCY
        421696 VIRUS
         49647 HUMAN IMMUNODEFICIENCY VIRUS
                 (HUMAN(W)IMMUNODEFICIENCY(W)VIRUS)
L34     169959 (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)

=> s l34 and (CpG)
          9510 CPG
L35        127 L34 AND (CPG)

=> s l35 and py<2000
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                 (PY<20000000)
L36         20 L35 AND PY<2000

=> s l36 and (ALUM or saponin)
          2175 ALUM
          3485 SAPONIN
L37          1 L36 AND (ALUM OR SAPONIN)

=> d l37,cbib,ab

L37  ANSWER 1 OF 1     MEDLINE on STN
1999279901.    PubMed ID: 10353461.   Immunostimulatory CpG motifs trigger a
     T helper-1 immune response to human immunodeficiency virus type-1
     (HIV-1) gp 160 envelope proteins. Deml L; Schirmbeck R; Reimann J; Wolf
     H; Wagner R. (Institute of Medical Microbiology, University of Regensburg,
     Germany. ) Clinical chemistry and laboratory medicine : CCLM / FESCC,
     (1999 Mar) Vol. 37, No. 3, pp. 199-204.  Journal code: 9806306. ISSN:
     1434-6621. Pub. country: GERMANY: Germany, Federal Republic of. Language:
     English.
AB   Bacterial DNA sequences containing unmethylated CpG motifs have recently
     been proposed to exhibit immunostimulatory effects on B-, T- and NK cells,
     leading to the induction of humoral and cell-mediated immune responses.
     In the present study we investigated the immunomodulatory effects of a
     CpG-containing oligodeoxynucleotide (CpG ODN) to the HIV-1 gp 160
     envelope (Env) protein in the BALB/c mouse model.  Priming and boosting of
     mice with gp 160 adsorbed to aluminium hydroxide (Alum) induced a
     typical T helper-2 (Th2)-dominated immune response with high titers of gp
     160-specific immunoglobulin (Ig)G1 isotypes but a weak IgG2a response.
     Specifically re-stimulated splenocytes from these mice predominantly
     secreted interleukin (IL)-5 but only minute amounts of interferon-gamma
     (IFN-gamma) upon specific re-stimulation.  In contrast, a boost
     immunisation of gp 160/Alum primed mice with a gp 160/Alum/CpG
     combination resulted in a seven times higher production of IgG2a
     antibodies, without affecting the titers of IgG1 isotypes.  Furthermore,
     approximately 10-fold increased levels of IFN-gamma, but significantly
     reduced amounts of IL-5, were secreted from gp 160-restimulated splenic
     cells.  A further greater than 30-fold increase in the levels of specific
```

IgG2a responses and a substantially elevated secretion of IFN-gamma were observed when the mice received gp160/**Alum**/**CpG** combinations for priming and boost injections. Thus, **CpG** ODNs are useful as an adjuvant to induce a typical Th0/Th1 response to **HIV** gp 160 proteins. However, despite the induction of a more Th1-like immune response, gp 160/**Alum**/**CpG** combinations were not sufficient to prime an Env-specific cytotoxic T-cell (CTL) response.

```
=> d his

     (FILE 'HOME' ENTERED AT 00:18:42 ON 13 NOV 2006)

     FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006
                E GARCON NATALIE/IN
L1            25 S E4-E7
L2             8 S L1 AND (CPG)
L3            17 S L1 NOT L2
                E FRIEDE MARTIN/IN
L4            17 S E3
L5            12 S L4 NOT L1
L6            12 S (WD1001 OR WD1002 OR WD1003 OR WD1004 OR WD1005 OR WD1006 OR
L7            11 S L6 NOT L1
L8            11 S L7 NOT L4
L9             1 S L8 AND (CPG)
L10          154 S (TCCATGACGTTCCTGACGTT)
L11          154 S L10 NOT L1
L12          154 S L11 NOT L4
L13          154 S L12 AND (CPG)
L14          138 S L13 AND (IMMUNOSTIMULATORY)
L15           12 S L14 AND AY<2000
L16          110 S (TCTCCCAGCGTGCGCCAT)
L17            8 S L16 AND L15
L18            1 S (ACCGATAACGTTGCCGGTGACG)
L19            9 S (TCCATGAGCTTCCTGAGCTT)
L20            8 S L19 AND L16
L21            0 S L20 AND AY<2000
L22            5 S (ACCGATGACGTCGCCGGTGACGGCACCACG)
L23            0 S L22 AND L15
L24        48505 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L25         1086 S L24 AND (GP120/CLM OR GP160/CLM OR ENV/CLM)
L26           14 S L25 AND (ALUM/CLM OR SAPONIN/CLM)
L27            3 S L26 AND (ALUM/CLM AND SAPONIN/CLM)
L28           11 S L26 NOT L27
L29            1 S L28 AND AY<2000

     FILE 'WPIDS' ENTERED AT 00:59:06 ON 13 NOV 2006
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L30           22 S E3-E6
L31            8 S L30 AND (HIV)
L32            2 S L31 AND (NEF)
L33            1 S L32 AND (PROTEIN D)

     FILE 'MEDLINE' ENTERED AT 01:02:04 ON 13 NOV 2006
L34       169959 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L35          127 S L34 AND (CPG)
L36           20 S L35 AND PY<2000
L37            1 S L36 AND (ALUM OR SAPONIN)

=> s l36 not l37
L38           19 L36 NOT L37

=> d l38,cbib,ab,1-19

L38  ANSWER 1 OF 19     MEDLINE on STN
1999355330.    PubMed ID: 10428376.   Molecular basis of HIV-1 TAR RNA
     specific recognition by an acridine tat-antagonist. Gelus N; Hamy F;
     Bailly C. (INSERM Unite 524, IRCL, Lille, France. ) Bioorganic & medicinal
     chemistry, (1999 Jun) Vol. 7, No. 6, pp. 1075-9.  Journal code: 9413298.
     ISSN: 0968-0896. Pub. country: ENGLAND: United Kingdom. Language: English.
AB   We investigated the interaction of a highly potent acridine-based
     tat-antagonist with the TAR RNA of HIV-1.  The wild type TAR RNA and
     three mutants with U-->C23, G x C-->C x G26-39 or G x C-->A x U26-39
     substitutions were used as substrates to study the molecular basis of
     drug-TAR RNA complex formation.  Melting temperature and RNase protection
     experiments reveal that the G x C26-39 pair is a critical element for
     specific major groove recognition of TAR at the pyrimidine bulge.  The
     results provide a rational basis for future design of optimized tat/TAR
     inhibitors.
```

L38 ANSWER 2 OF 19 MEDLINE on STN

1999294416. PubMed ID: 10367950. Gene gun DNA vaccination with Rev-independent synthetic **HIV**-1 gp160 envelope gene using mammalian codons. Vinner L; Nielsen H V; Bryder K; Corbet S; Nielsen C; Fomsgaard A. (Department of Virology, Statens Serum Institut, Copenhagen, Denmark.) Vaccine, **(1999 Apr 23)** Vol. 17, No. 17, pp. 2166-75. Journal code: 8406899. ISSN: 0264-410X. Pub. country: ENGLAND: United Kingdom. Language: English.

AB DNA immunization with **HIV** envelope plasmids induce only moderate levels of specific antibodies which may in part be due to limitations in expression influenced by a species-specific and biased **HIV** codon usage. We compared antibody levels, Th1/Th2 type and CTL responses induced by synthetic genes encoding membrane bound gp160 versus secreted gp120 using optimized codons and the efficient gene gun immunization method. The in vitro expression of syn.gp160 as gp120 + gp41 was Rev independent and much higher than a classical wt.gp160 plasmid. Mice immunized with syn.gp160 and wt.gp160 generated low and inconsistent ELISA antibody titres whereas the secreted gp120 consistently induced faster seroconversion and higher antibody titres. Due to a higher C + G content the numbers of putative **CpG** immune (Th1) stimulatory motifs were highest in the synthetic gp160 gene. However, both synthetic genes induced an equally strong and more pronounced Th2 response with higher IgG1/IgG2a and IFNgamma/IL-4 ratios than the wt.gp160 gene. As for induction of CTL, synthetic genes induced a somewhat earlier response but did not offer any advantage over wild type genes at a later time point. Thus, optimizing codon usage has the advantage of rendering the structural **HIV** genes Rev independent. For induction of antibodies the level of expression, while important, seems less critical than optimal contact with antigen presenting cells at locations reached by the secreted gp120 protein. A proposed Th1 adjuvant effect of the higher numbers of **CpG** motifs in the synthetic genes was not seen using gene gun immunization which may be due to the low amount of DNA used.

L38 ANSWER 3 OF 19 MEDLINE on STN

1999191219. PubMed ID: 10091119. Major versus minor groove DNA binding of a bisarginylporphyrin hybrid molecule: a molecular mechanics investigation. Gresh N; Perree-Fauvet M. (Laboratoire de Pharmacochimie Moleculaire et Structurale, CNRS-URA 1500, INSERM U266, Universite Paris 5, France.) Journal of computer-aided molecular design, **(1999 Mar)** Vol. 13, No. 2, pp. 123-37. Journal code: 8710425. ISSN: 0920-654X. Pub. country: Netherlands. Language: English.

AB On the basis of theoretical computations, we have recently synthesised [Perree-Fauvet, M. and Gresh, N., Tetrahedron Lett., 36 (1995) 4227] a bisarginyl conjugate of a tricationic porphyrin (BAP), designed to target, in the major groove of DNA, the d(GGC GCC)2 sequence which is part of the primary binding site of the **HIV**-1 retrovirus site [Wain-Hobson, S. et al., Cell, 40 (1985) 9]. In the theoretical model, the chromophore intercalates at the central d(**CpG**)2 step and each of the arginyl arms targets O6/N7 belonging to guanine bases flanking the intercalation site. Recent IR and UV-visible spectroscopic studies have confirmed the essential features of these theoretical predictions [Mohammadi, S. et al., Biochemistry, 37 (1998) 6165]. In the present study, we compare the energies of competing intercalation modes of BAP to several double-stranded oligonucleotides, according to whether one, two or three N-methylpyridinium rings project into the major groove. Correspondingly, three minor groove binding modes were considered, the arginyl arms now targeting N3, O2 sites belonging to the purine or pyrimidine bases flanking the intercalation site. This investigation has shown that: (i) in both the major and minor grooves, the best-bound complexes have the three N-methylpyridinium rings in the groove opposite to that of the phenyl group bearing the arginyl arms; (ii) major groove binding is preferred over minor groove binding by a significant energy (29 kcal/mol); and (iii) the best-bound sequence in the major groove is d(GGC GCC)2 with two successive guanines upstream from the intercalation. On the other hand, due to the flexibility of the arginyl arms, other GC-rich sequences have close binding energies, two of them being less stable than it by less than 8 kcal/mol. These results serve as the basis for the design of derivatives of BAP with enhanced sequence selectivities in the major groove.

L38 ANSWER 4 OF 19 MEDLINE on STN

1999091709. PubMed ID: 9873082. Biased nucleotide composition of the genome of HERV-K related endogenous retroviruses and its evolutionary implications. Zsiros J; Jebbink M F; Lukashov V V; Voute P A; Berkhout B. (Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.) Journal of molecular evolution, **(1999 Jan)** Vol. 48, No. 1, pp. 102-11. Journal code: 0360051. ISSN: 0022-2844. Pub. country: United States. Language:

English.

AB The human genome contains a large number of sequences that belong to the HERV-K family of human endogenous retroviruses. Most of these elements are likely remnants of ancient infections by ancestral exogenous retroviruses. To obtain further insight into the evolutionary history and molecular mechanisms responsible for the diversity of the human HERV-K elements, we analyzed several aspects of their genome structure. The nucleotide composition of the HERV-K genome was found to be highly biased and asymmetric, with an abundance of the A nucleotide in the viral (+) strand. A similar trend has been reported for the genomes of several exogenous retroviruses, with different nucleotides as the preferred building block. Other genome characteristics that were reported previously for actively replicating retroviruses are also apparent for the endogenous HERV-K virus. In particular, we observed suppression of the dinucleotide **CpG**, which represents potential methylation sites, and a strong preference for synonymous substitutions within the open reading frame of the reverse transcriptase (RT) enzyme. Furthermore, the mutational spectrum of the HERV-K RT enzyme was evaluated by nucleotide sequence comparison of 34 available elements. Interestingly, this analysis revealed a striking similarity with the mutational pattern of the **HIV**-1 RT enzyme, with a preference for G-to-A and C-to-T transitions. It is proposed that the mutational bias of the HERV-K RT enzyme played a role in the shaping of this retroviral genome, which was actively replicating more than 30 million years ago. This effect can still be observed in the contemporary endogenous HERV-K elements.

L38 ANSWER 5 OF 19 MEDLINE on STN

1998378529. PubMed ID: 9710601. Infection with **human immunodeficiency virus** type 1 upregulates DNA methyltransferase, resulting in de novo methylation of the gamma interferon (IFN-gamma) promoter and subsequent downregulation of IFN-gamma production. Mikovits J A; Young H A; Vertino P; Issa J P; Pitha P M; Turcoski-Corrales S; Taub D D; Petrow C L; Baylin S B; Ruscetti F W. (Intramural Research Support Program, SAIC Frederick, Division of Basic Sciences, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick Maryland 21702-1201, USA.. Mikovits@fcrfv1.ncifcrf.gov) . Molecular and cellular biology, **(1998 Sep)** Vol. 18, No. 9, pp. 5166-77. Journal code: 8109087. ISSN: 0270-7306. Pub. country: United States. Language: English.

AB The immune response to pathogens is regulated by a delicate balance of cytokines. The dysregulation of cytokine gene expression, including interleukin-12, tumor necrosis factor alpha, and gamma interferon (IFN-gamma), following human retrovirus infection is well documented. One process by which such gene expression may be modulated is altered DNA methylation. In subsets of T-helper cells, the expression of IFN-gamma, a cytokine important to the immune response to viral infection, is regulated in part by DNA methylation such that mRNA expression inversely correlates with the methylation status of the promoter. Of the many possible genes whose methylation status could be affected by viral infection, we examined the IFN-gamma gene as a candidate. We show here that acute infection of cells with **human immunodeficiency virus** type 1 (**HIV**-1) results in (i) increased DNA methyltransferase expression and activity, (ii) an overall increase in methylation of DNA in infected cells, and (iii) the de novo methylation of a **CpG** dinucleotide in the IFN-gamma gene promoter, resulting in the subsequent downregulation of expression of this cytokine. The introduction of an antisense methyltransferase construct into lymphoid cells resulted in markedly decreased methyltransferase expression, hypomethylation throughout the IFN-gamma gene, and increased IFN-gamma production, demonstrating a direct link between methyltransferase and IFN-gamma gene expression. The ability of increased DNA methyltransferase activity to downregulate the expression of genes like the IFN-gamma gene may be one of the mechanisms for dysfunction of T cells in **HIV**-1-infected individuals.

L38 ANSWER 6 OF 19 MEDLINE on STN

1998334516. PubMed ID: 9667948. Use of Nalpha-Fmoc-cysteine(S-thiobutyl) derivatized oligodeoxynucleotides for the preparation of oligodeoxynucleotide-peptide hybrid molecules. Soukchareun S; Haralambidis J; Tregear G. (The Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria 3052, Australia.. sommay@hermes.scripps.edu) . Bioconjugate chemistry, **(1998 Jul-Aug)** Vol. 9, No. 4, pp. 466-75. Journal code: 9010319. ISSN: 1043-1802. Pub. country: United States. Language: English.

AB The chemical modification of antisense oligodeoxynucleotides (ODNs) by conjugating synthetic peptides of known membranotropic activities to the 3' and/or 5' terminus of ODNs may serve two functions that are important for increasing their bioavailability by protecting the ODNs from exonuclease digestion and facilitated delivery into cells. We have previously reported the preparation of ODN-peptide conjugates by the total synthesis approach. However, by such technology the preparation of

ODN-peptide conjugates in amounts sufficient for in vitro functional analysis is at present limited to the syntheses of peptides containing residues without acidolytic deprotection. Requisite to the alternative method of site-specific conjugation, the segment coupling approach is the derivatization of an ODN with a nucleophilic moiety. In this paper, we describe a novel method of functionalizing synthetic ODNs by incorporating S-thiobutyl-protected Nalpha-Fmoc-cysteine to aminopropyl-functionalized **CPG** by standard Nalpha-Fmoc SPPS methodology. The derivatized solid support can be used to synthesize an ODN of any sequence by the phosphoramidite chemistry, and the removal of the S-thiobutyl side chain function can be conveniently affected by the standard amminolytic deprotection of ODNs containing 1 M DTT. The purified cysteine-derivatized ODN was shown to react specifically and efficiently with two types of synthetic peptides corresponding to regions within the glycoprotein (gp) of **HIV** that have been shown to have membranotropic activities: a 18 residue maleimide-derivatized Tat peptide of the transactivator (tat) of **HIV** and a 22 residue peptide corresponding to the carboxyl terminus of gp41(Ca-gp41).

L38 ANSWER 7 OF 19 MEDLINE on STN

1998227680. PubMed ID: 9568729. IFN-gamma primes macrophage responses to bacterial DNA. Sweet M J; Stacey K J; Kakuda D K; Markovich D; Hume D A. (Centre for Molecular and Cellular Biology, Department of Microbiology, University of Queensland, Brisbane, Australia.) Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research, **(1998 Apr)** Vol. 18, No. 4, pp. 263-71. Journal code: 9507088. ISSN: 1079-9907. Pub. country: United States. Language: English.

AB Macrophages recognize and are activated by unmethylated **CpG** motifs in bacterial DNA. Here we demonstrate that production of nitric oxide (NO) from murine RAW 264 macrophages and bone marrow-derived macrophages (BMM) in response to bacterial DNA is absolutely dependent on interferon-gamma (IFN-gamma) priming. Similarly, arginine uptake and expression of the inducible nitric oxide synthase (iNOS) gene in response to bacterial DNA in BMM occurred only after IFN-gamma priming. In contrast, mRNA for the cationic amino acid transporter, CAT2, was induced by plasmid DNA alone, and priming with IFN-gamma had no effect on this response. Tumor necrosis factor-alpha (TNF-alpha) release from RAW 264 and BMM in response to bacterial DNA was augmented by IFN-gamma pretreatment. In a stably transfected **HIV**-1 long terminal repeat (LTR) luciferase RAW 264 cell line, IFN-gamma and bacterial DNA synergized in activation of the **HIV**-1 LTR. Bacterial DNA has been shown to induce IFN-gamma production in vivo as an indirect consequence of interleukin-12 (IL-12) and TNF-alpha production from macrophages. The results herein suggest the existence of a self-amplifying loop that may have implications for therapeutic applications of bacterial DNA.

L38 ANSWER 8 OF 19 MEDLINE on STN

1998226658. PubMed ID: 9558356. Joint molecular modeling and spectroscopic studies of DNA complexes of a bis(arginyl) conjugate of a tricationic porphyrin designed to target the major groove. Mohammadi S; Perree-Fauvet M; Gresh N; Hillairet K; Taillandier E. (Laboratoire de Chimie Structurale et Spectroscopie Biomoleculaire (CNRS-URA 1430), Universite Paris 13, Bobigny, France.) Biochemistry, **(1998 Apr 28)** Vol. 37, No. 17, pp. 6165-78. Journal code: 0370623. ISSN: 0006-2960. Pub. country: United States. Language: English.

AB To target selectively the major groove of double-stranded B DNA, we have designed and synthesized a bis(arginyl) conjugate of a tricationic porphyrin (BAP). Its binding energies with a series of double-stranded dodecanucleotides, having in common a central d(**CpG**)2 intercalation site were compared. The theoretical results indicated a significant energy preference favoring major groove over minor groove binding and a preferential binding to a sequence encompassing the palindrome GGCGCC encountered in the Primary Binding Site of the **HIV**-1 retrovirus. Spectroscopic studies were carried out on the complexes of BAP with poly(dG-dC) and poly(dA-dT) and a series of oligonucleotide duplexes having either a GGCGCC, CCCGGG, or TACGTA sequence. The results of UV-visible and circular dichroism spectroscopies indicated that intercalation of the porphyrin takes place in poly(dG-dC) and all the oligonucleotides. Thermal denaturation studies showed that BAP increased significantly the melting temperature of the oligonucleotides having the GGCGCC sequence, whereas it produced only a negligible stabilization of sequences having CCCGGG or TACGTA in place of GGCGCC. This indicates a preferential binding of BAP to GGCGCC, fully consistent with the theoretical predictions. IR spectroscopy on d(GGCGCC)2 indicated that the guanine absorption bands, C6=O6 and N7-C8-H, were shifted by the binding of BAP, indicative of the interactions of the arginine arms in the major groove. Thus, the de novo designed compound BAP constitutes one of the very rare intercalators which, similar to the antitumor drugs mitoxantrone

and ditercalinium, binds DNA in the major groove rather than in the minor groove.

L38 ANSWER 9 OF 19 MEDLINE on STN
96417780. PubMed ID: 8820571. Lymphocyte activation by **CpG** dinucleotide motifs in prokaryotic DNA. Krieg A M. (Department of Internal Medicine, University of Iowa College of Medicine, Iowa City 52246, USA.. arthur-krieg@uniowa.edu) . Trends in microbiology, **(1996 Feb)** Vol. 4, No. 2, pp. 73-6. Ref: 35. Journal code: 9310916. ISSN: 0966-842X. Pub. country: ENGLAND: United Kingdom. Language: English.
AB **CpG** dinucleotides are present at the expected frequency in prokaryotic DNA, but are underrepresented ('**CpG** suppression') and methylated in vertebrate DNA. The vertebrate immune system has apparently evolved the ability to recognize these unmethylated **CpG** motifs and respond with a rapid and coordinated cytokine response leading to the induction of humoral and cell-mediated immunity.

L38 ANSWER 10 OF 19 MEDLINE on STN
96377832. PubMed ID: 8783641. Amplification of antibody production by phosphorothioate oligodeoxynucleotides. Branda R F; Moore A L; Lafayette A R; Mathews L; Hong R; Zon G; Brown T; McCormack J J. (Genetics Laboratory, University of Vermont, Burlington 05401, USA.) The Journal of laboratory and clinical medicine, **(1996 Sep)** Vol. 128, No. 3, pp. 329-38. Journal code: 0375375. ISSN: 0022-2143. Pub. country: United States. Language: English.
AB A phosphorothioate oligodeoxynucleotide that is complementary (antisense) to the initiation region of the rev gene of **HIV**-1 causes hypergammaglobulinemia and splenomegaly in mice, and it induces B cell proliferation and differentiation in mouse spleen mononuclear cells (SMNCs) and human peripheral blood mononuclear cells in vitro. The current studies were performed to investigate the specificity of these immunomodulatory effects. Both the sense and antisense rev oligomers stimulated tritiated thymidine incorporation and secretion of immunoglobulin M (IgM) and immunoglobulin G (IgG) by mouse SMNCs in a concentration-dependent fashion, but the antisense oligomer produced greater immune effects. Studies comparing phosphorothioate oligomers (anti-rev, c-myc, and c-myb) either methylated or unmethylated at **CpG** dinucleotides showed that methylation effectively abrogated the proliferative effect and tended to reduce the immunoglobulin secretory activity, but the latter was not statistically significant except in the case of IgG in anti-rev oligomer-treated cultures. Mice were injected with the sense or antisense rev oligomers singly or in combination. The animals then were immunized with tetanus toxoid and received a booster 21 days later. Oligodeoxynucleotide-treated mice had significantly higher levels of IgM antibodies on days 28 and 35 and of IgG antibodies on days 14 and 35 as compared with mice that were immunized but received vehicle alone. There was no evidence for additive, synergistic, or antagonistic interactions of the sense and antisense rev oligomers. These results indicate that the unmethylated anti-rev oligomer is the most potent of the phosphorothioate oligomers tested at activating lymphocyte proliferation and differentiation and that a single intravenous injection of this oligodeoxynucleotide augments antibody production to a specific antigen as long as 35 days later.

L38 ANSWER 11 OF 19 MEDLINE on STN
96355018. PubMed ID: 8757335. Macrophages ingest and are activated by bacterial DNA. Stacey K J; Sweet M J; Hume D A. (Centre for Molecular and Cellular Biology, University of Queensland, Brisbane, Australia.) Journal of immunology (Baltimore, Md. : 1950), **(1996 Sep 1)** Vol. 157, No. 5, pp. 2116-22. Journal code: 2985117R. ISSN: 0022-1767. Pub. country: United States. Language: English.
AB Recent evidence suggests that bacterial DNA activates immune responses. Here we showed that TNF-alpha mRNA was induced in bone marrow-derived macrophages and the macrophage cell line RAW 264 by plasmid DNA, but not by DNaseI-digested plasmid, plasmid methylated on **CpG** dinucleotides, or by vertebrate genomic DNA, which is naturally largely methylated on these sequences. Synthetic polynucleotides poly d(I-C) and poly I x poly C also induced TNF-alpha. IL-1 beta and plasminogen activator inhibitor-2 mRNAs were induced by plasmid DNA, and IFN-gamma-pretreated macrophages responded to DNA with induction of inducible nitric oxide synthase. The **HIV**-1 long terminal repeat was activated by exogenous DNA in a manner similar to TNF-alpha, and was also activated by a **CpG**-containing oligonucleotide. Transcription factor nuclear factor-kappa B (NF-kappa B) is involved in regulation of the **HIV**-1 long terminal repeat and many inflammatory response genes. NF-kappa B binding activity was increased by plasmid DNA. An important question is whether these effects involve DNA binding to a cell surface receptor that signals to the interior, or whether internalization is necessary. Here we found that plasmid was taken up by RAW 264 cells and remained sufficiently intact to code for

luciferase protein. Results suggest that DNA is taken up by macrophages and characteristic bacterial DNA sequences, which include an unmethylated **CpG** sequence, activate a signaling cascade leading to activation of NF-kappa B and inflammatory gene induction. Relevance to DNA vaccination, gene therapy, antisense, and transfection studies is discussed.

L38 ANSWER 12 OF 19 MEDLINE on STN

95222722. PubMed ID: 7707499. Molecular and functional interactions of transcription factor USF with the long terminal repeat of **human immunodeficiency virus** type 1. d'Adda di Fagagna F; Marzio G; Gutierrez M I; Kang L Y; Falaschi A; Giacca M. (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy.) Journal of virology, **(1995 May)** Vol. 69, No. 5, pp. 2765-75. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB The human transcription factor USF, purified from HeLa cells, and its recombinant 43-kDa component bind to the long terminal repeat (LTR) of **human immunodeficiency virus** type 1. The proteins footprint over nucleotides from position -173 to -157 upstream of the transcription start site, generating strong DNAse I hypersensitivity sites at the 3' sides on both strands. As detected by methylation protection studies, the factor forms symmetric contacts with the guanines of the palindromic CACGTG core of the recognized sequence. Its binding ability is abolished by the mutation of this core sequence and is strongly reduced by the cytosine methylation of the central **CpG** dinucleotide. Upon binding, both recombinant and purified USFs bend the LTR DNA template. The role of USF in the control of transcription initiation from the LTR was tested by in vitro transcription assays. Upon addition of the protein, transcription from constructs containing an intact binding site is increased, while the responsiveness in constructs with a mutated sequence is abolished. Furthermore, the addition of a decoy plasmid which contains multiple repeats of the target sequence results in downregulation of transcription from the LTR. These results suggest that USF is a positive regulator of LTR-mediated transcriptional activation.

L38 ANSWER 13 OF 19 MEDLINE on STN

93267388. PubMed ID: 8496786. Transcription of the **HIV**-1 LTR is regulated by the density of DNA **CpG** methylation. Gutekunst K A; Kashanchi F; Brady J N; Bednarik D P. (Centers for Disease Control, Retrovirus Diseases Branch, Atlanta, Georgia.) Journal of acquired immune deficiency syndromes, **(1993 Jun)** Vol. 6, No. 6, pp. 541-9. Journal code: 8812597. ISSN: 0894-9255. Pub. country: United States. Language: English.

AB Transcription from the **HIV**-1 long terminal repeat (LTR) was shown to be inhibited by DNA **CpG** methylation both in vivo and in vitro. Enzymatic methylation of **CpG** sites localized within the LTR decreased the transcription of the CAT reporter gene, chloramphenicol acetyltransferase, as assayed by the transient expression of this gene in tissue culture. The inhibitory effect could be initially overcome, in trans, by the transactivator tat. As a function of time, the presence of tat had no observable effect on transcription, within the limits of detection sensitivity, suggesting that the level of basal transcription was reduced to very low levels. This effect is suggestive of the involvement of cellular **CpG** methylation-dependent inhibitory factors which have been characterized by other laboratories. These data imply that transactivation is reduced to low levels after longer periods of time when the DNA template is sparsely methylated. The transcriptional inhibitory process may involve proteins such as MeCP which may interact with methylated DNA more slowly and/or weakly. Conversely, densely methylated DNA was transcriptionally repressed immediately which suggests the rapid/strong association of the cellular inhibitory factor(s). The transcriptional inhibitory effect was also observed in an in vitro transcription run-off system. These data suggest that the methylation-mediated inhibition of transcription is directly affected by **CpG** methylation density and may involve other factors.

L38 ANSWER 14 OF 19 MEDLINE on STN

92334993. PubMed ID: 1630912. Synthesis and physical properties of anti-**HIV** antisense oligonucleotides bearing terminal lipophilic groups. MacKellar C; Graham D; Will D W; Burgess S; Brown T. (Department of Chemistry, University of Edinburgh, UK.) Nucleic acids research, **(1992 Jul 11)** Vol. 20, No. 13, pp. 3411-7. Journal code: 0411011. ISSN: 0305-1048. Pub. country: ENGLAND: United Kingdom. Language: English.

AB A number of phosphoramidite monomers have been prepared and used in the synthesis of antisense phosphorothioate oligonucleotides bearing 5'-polyalkyl and cholesterol moieties. Similar groups have also been attached to the 3'-end of oligonucleotides by means of functionalised **CPG**. Melting temperatures of duplexes formed between phosphorothioate oligonucleotides with lipophilic end-groups and complementary DNA strands were found to be identical to those formed by the equivalent unmodified

phosphorothioates.

L38 ANSWER 15 OF 19 MEDLINE on STN
92118717. PubMed ID: 1768651. DNA **CpG** methylation inhibits binding of NF-kappa B proteins to the **HIV**-1 long terminal repeat cognate DNA motifs. Bednarik D P; Duckett C; Kim S U; Perez V L; Griffis K; Guenthner P C; Folks T M. (Centers for Disease Control, Division of Viral and Rickettsial Diseases, Atlanta, GA 30333.) The New biologist, **(1991 Oct)** Vol. 3, No. 10, pp. 969-76. Journal code: 9000976. ISSN: 1043-4674. Pub. country: United States. Language: English.
AB The regulation of cellular or viral gene expression is directly influenced by the pattern of methylated cytosine residues localized in the DNA of enhancer/promoter sequences. The mechanism of transcriptional silencing has been explained on the basis of either an indirect model, in which densely methylated DNA is recognized by proteins that may displace crucial transcription factors, or a direct model, in which binding of a single transcription protein is prevented by the presence of a methylated **CpG** dinucleotide localized in a sensitive region of a DNA motif. In this study, we have determined that methylation of the core **CpG** dinucleotide located within the NF-kappa B repeated motifs of the **human immunodeficiency virus** type 1 (**HIV**-1) long terminal repeat can inhibit the binding of the NF-kappa B protein complex from crude nuclear extracts or from purified bovine spleen and specifically inhibit the binding of recombinant p50 protein. We have used the electrophoretic mobility shift assay (EMSA) and DNaseI footprinting analysis to demonstrate that binding of the NF-kappa B proteins to their cognate motifs can be inhibited via the direct model proposed for methylation-mediated inhibition of DNA-protein interaction.

L38 ANSWER 16 OF 19 MEDLINE on STN
91374590. PubMed ID: 1654446. Identification of a transactivating function mapping to the putative immediate-early locus of human herpesvirus 6. Martin M E; Nicholas J; Thomson B J; Newman C; Honess R W. (Division of Virology, National Institute for Medical Research, London, United Kingdom.) Journal of virology, **(1991 Oct)** Vol. 65, No. 10, pp. 5381-90. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.
AB Sequencing studies have indicated that the unique component of the human herpesvirus 6 (HHV-6) genome and the unique long segment of the human cytomegalovirus genome are genetically colinear. Of particular interest is the identification of a region of local **CpG** dinucleotide suppression in the genome of HHV-6, a feature conserved in the genomes of human cytomegalovirus, murine cytomegalovirus, and simian cytomegalovirus, and a characteristic of the major immediate-early loci of these viruses. Adjacent to this region in HHV-6 are approximately 30 copies of a 103- to 108-bp sequence element, which contains consensus binding sites for the transcription factors AP2 and NF kappa B, in addition to a single KpnI recognition site. Together, these KpnI repeat units may compose an immediate-early enhancer, analogous to those found in the cytomegaloviruses. We present the sequence of this region of HHV-6 and demonstrate that a transactivating function is encoded by this region. We have used polymerase chain reaction to synthesize fragments containing open reading frames and 5' sequences with or without the upstream KpnI repeat units. Effector plasmids containing these HHV-6 coding and 5' sequences were able to effect activation of heterologous promoter-chloramphenicol acetyltransferase (CAT) constructs, including adenovirus E3-CAT and E4-CAT, human T-cell lymphotropic virus type I long terminal repeat (LTR)-CAT, and **human immunodeficiency virus** LTR-CAT, in cotransfection experiments in Vero cells and peripheral blood lymphocytes. Furthermore, we have identified the major open reading frame (RF4; 2.3 kb) as being essential for activation, and we have shown that the NF kappa B, SP1, and TATA box motifs in the **human immunodeficiency virus** LTR are all required for full induction of the promoter by the HHV-6-encoded transactivator.

L38 ANSWER 17 OF 19 MEDLINE on STN
90214625. PubMed ID: 2323336. Inactivation of the **HIV** LTR by DNA **CpG** methylation: evidence for a role in latency. Bednarik D P; Cook J A; Pitha P M. (Johns Hopkins University, School of Medicine Oncology Center, Baltimore, MD 21205.) The EMBO journal, **(1990 Apr)** Vol. 9, No. 4, pp. 1157-64. Journal code: 8208664. ISSN: 0261-4189. Pub. country: ENGLAND: United Kingdom. Language: English.
AB Infection of cells by **HIV** can result in a period of quiescence or latency which may be obviated by treatment with inducing agents such as 5-azacytidine. Evidence from these experiments demonstrate the existence of two **CpG** sites in the **HIV** LTR which can silence transcription of both reporter genes (CAT) and infectious proviral DNA when enzymatically methylated. This transcriptional block was consistently overcome by the presence of the trans-activator tat without significant demethylation of

the **HIV** LTR. These results suggest that DNA hypermethylation of the **HIV** LTR may change the binding characteristics between LTR sequences and cellular proteins, thereby suppressing **HIV** LTR transcription and modulating viral expression.

L38 ANSWER 18 OF 19 MEDLINE on STN
90116943. PubMed ID: 2692125. Are retroviruses involved in the pathogenesis of SLE? Evidence demonstrated by molecular analysis of nucleic acids from SLE patients' plasma. Krapf F E; Herrmann M; Leitmann W; Kalden J R. (IIIrd Department for Internal Medicine, University Erlangen-Nurnberg, Federal Republic of Germany.) Rheumatology international, **(1989)** Vol. 9, No. 3-5, pp. 115-21. Journal code: 8206885. ISSN: 0172-8172. Pub. country: GERMANY, WEST: Germany, Federal Republic of. Language: English.
AB High molecular weight DNA of up to 20 kbp and, additionally, an RNase-insensitive RNA of more than 60 b were isolated from plasmapheresis fluids taken from patients with active systemic lupus erythematosus (SLE). Similar nucleic acids could not be demonstrated in the plasma samples from patients with Waldenstroem's disease, rheumatoid arthritis, myasthenia gravis, and other diseases including active systemic disorders. The purified nucleic acids were analyzed in several ways; they proved to be immunogenic by inducing polyclonal and monoclonal antibodies to natural DNA as well as to synthetic polynucleotides (e.g. polyguanylic acid) after injection into experimental animals (rabbits or mice respectively). Biochemical and molecular cloning analysis of the DNA revealed features like high levels of **CpG**-dinucleotides, usually not observed in common human DNA. A possible exogenous origin was substantiated by comparative sequence studies of cloned plasma DNA, which showed homologies to human retroviruses, e.g. PL1 (85%/60 b) and the sequences of the gag and pol genes of **human immunodeficiency virus** type I (85%/157 b). Experiments applying isolated plasma nucleic acids in transfection experiments showed the induction of morphological changes in an EBV-immortalized B-cell line drawn from a healthy human donor, such as vacuolization and syncitia formation. Northern blot analysis demonstrated, exclusively in the transfected cell line, the expression of mRNA homologous to the cloned plasma DNA. Using this clone as a probe, homologous sequences could be demonstrated by Northern blot analysis in EBV-immortalized cell lines from SLE patients only and, by means of DNA amplification, in peripheral blood lymphocytes from SLE and AIDS patients.(ABSTRACT TRUNCATED AT 250 WORDS)

L38 ANSWER 19 OF 19 MEDLINE on STN
90089402. PubMed ID: 2597678. Nucleotide composition bias and **CpG** dinucleotide content in the genomes of **HIV** and HTLV 1/2. Kypr J; Mrazek J; Reich J. (Institute of Biophysics, Czechoslovak Academy of Sciences, Brno.) Biochimica et biophysica acta, **(1989 Dec 22)** Vol. 1009, No. 3, pp. 280-2. Journal code: 0217513. ISSN: 0006-3002. Pub. country: Netherlands. Language: English.
AB Nucleotide compositions of the **HIV** subfamily and HTLV 1/2 genomes are strongly biased in a remarkably opposite way; **HIV** is adenine-rich and cytosine-poor while HTLV 1/2 is cytosine-rich and adenine-poor. In addition, the **CpG** dinucleotides are underrepresented in **HIV** but abundant in HTLV 1/2. By these two properties the genomes of **HIV** and HTLV 1/2 mimic an (A + T)-rich and (G + C)-rich segment of the host genome, respectively. These dramatic differences between the two human retroviruses might have evolved to direct integration of the retroviral genomes into specific segments of the human chromosomes.

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FILE 'USPATFULL' ENTERED AT 00:18:51 ON 13 NOV 2006
E GARCON NATALIE/IN
L1 25 S E4-E7
L2 8 S L1 AND (CPG)
L3 17 S L1 NOT L2
E FRIEDE MARTIN/IN
L4 17 S E3
L5 12 S L4 NOT L1
L6 12 S (WD1001 OR WD1002 OR WD1003 OR WD1004 OR WD1005 OR WD1006 OR
L7 11 S L6 NOT L1
L8 11 S L7 NOT L4
L9 1 S L8 AND (CPG)
L10 154 S (TCCATGACGTTCCTGACGTT)
L11 154 S L10 NOT L1
L12 154 S L11 NOT L4
L13 154 S L12 AND (CPG)

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L15           12 S L14 AND AY<2000
L16          110 S (TCTCCCAGCGTGCGCCAT)
L17            8 S L16 AND L15
L18            1 S (ACCGATAACGTTGCCGGTGACG).
L19            9 S (TCCATGAGCTTCCTGAGCTT)
L20            8 S L19 AND L16
L21            0 S L20 AND AY<2000
L22            5 S (ACCGATGACGTCGCCGGTGACGGCACCACG)
L23            0 S L22 AND L15
L24        48505 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L25         1086 S L24 AND (GP120/CLM OR GP160/CLM OR ENV/CLM)
L26           14 S L25 AND (ALUM/CLM OR SAPONIN/CLM)
L27            3 S L26 AND (ALUM/CLM AND SAPONIN/CLM)
L28           11 S L26 NOT L27
L29            1 S L28 AND AY<2000

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L32            2 S L31 AND (NEF)
L33            1 S L32 AND (PROTEIN D)

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L36           20 S L35 AND PY<2000
L37            1 S L36 AND (ALUM OR SAPONIN)
L38           19 S L36 NOT L37

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ALL L# QUERIES AND ANSWER SETS ARE DELETED AT LOGOFF
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